**Introduction**

Wound is an injury to living tissue resulting in the loss of epithelial integrity accompanied by disruption of normal structure and function of the skin and its underlying tissues (Broughton et al., 2006; Senthil Kumar et al., 2006; Friedman, 2011). In response to wound, the physiological process of healing immediately sets in for restoring tissue integrity through specific immune response, termed as wound healing (Crovetti et al., 2004). Wound healing is a complex, precisely coordinated interplay involving inflammatory cells, cytokines and ECM components. The classic model of wound healing is divided into four sequential, yet overlapping phases that include hemostasis, inflammatory, proliferative and remodeling phase (Stadelmann et al., 1998). During these phases, significantly important processes such as formation of fibrin clot, establishment of immune barrier, dissolution of fibrin clot, removal of dead tissue, angiogenesis, ECM synthesis and development of new epithelium occur (Stroncek et al., 2009).

Wound care and management through the use of naturally derived agents has gained importance for their ability to reduce the time required for healing and minimizing the complications of infections (Hart, 2002). Among natural agents, plants have an immense potential for the management and treatment of wounds and have been used by tribal and folklore practitioners in many countries for the treatment of wounds; the property attributed to presence of various health sustaining bioactive constituents (Thakur et al., 2011; Korpenwar, 2012). Further the bioactive constituents augment the physiological actions on the human body to restore the structural and functional status of the damaged tissues (Singh et al., 2006). These constituents include various secondary metabolites, essential oils, and latex from few angiospermic plants (Edeoga et al., 2005). Particularly, latex preparations from various plants are topically applied on fresh wounds to stop bleeding and to hasten the process of wound healing (Thankamma, 2003). Further, the plant latex proteases such as papain, chymopapain (*Carica papaya*), plumerin-R (*Plumeria rubra*) and curcain (*Jatropha curcas*) have been reported to significantly enhance wound healing (Nath and Dutta, 1991; Enoch and Harding, 2003; Badgujar and Mahajan, 2011). However, the detailed molecular mechanisms by which these proteases exhibit wound healing activity are unclear. We have previously demonstrated that proteases isolated from
latex possess potent clot forming and clot dissolving properties. Further, earlier studies have also demonstrated the ability of latex proteases to mediate the decisive events of wound healing process (Rajesh et al., 2006; Shivaprasad et al., 2010).

*W. tinctoria* is a small, deciduous medicinal plant widely used by folk medicinal practitioners and tribal communities in various districts of Southern and Central states of India, for its immunomodulatory, anti-inflammatory, antinociceptive, antihemorrhagic, antipsoriatic, hepatoprotective and antidiarrhoeal properties (Bhosle et al., 2009; Rao and Sunitha, 2011). Latex from *W. tinctoria* is used for treatment of various clinical conditions including psoriasis, mouth ulcers, tooth ache, cough, fever and widely employed extensively for topical application on fresh wounds and blisters to promote healing (Murugesan et al., 2005).

Therefore, in the present study, wound healing activity of *W. tinctoria* latex proteases (WTLP) was studied by evaluating the physical, biochemical and histological parameters, which will provide scientific validation for the use of *W. tinctoria* plant latex as wound healing agent. The results demonstrate that, WTLP contains number of thermostable proteases that belongs to serine super family of proteases. Further, topical application of WTLP significantly enhanced healing of full thickness wound in murine model to support the traditional topical application of *W. tinctoria* after wound.

**Materials and methods**

*Collection of W. tinctoria latex and processing for protease fraction*

*W. tinctoria* R.Br. (Apocyanaceae) latex was processed as mentioned in methods section of chapter 2.

**Animals**

Adult Swiss Albino mice (30–35 g; either gender) were obtained from the Central Animal House Facility, UOM and were maintained in surface sterilized polypropylene cages with 12 h light dark cycle. The animal care and experimental procedures performed were in compliance with the Regulations for Animal Research and Animal Ethical Committee of the University of Mysore (Sanction order No. MGZ/2518(b)/2009-10), Mysore, India.
Chemicals

Ketamine and xylocaine were purchased from the University Medical facility with a prescription from the University authorized medical practitioner. Collagen type-I (from rat tail), collagen type-IV (from human placenta) and pepstatin A (from microbial source) were procured from Sigma Chemicals (St. Louis, MO, USA). PMSF, EDTA, IAA and p-DMAB were from Sisco Research Laboratory (Mumbai, India). Gelatin and Neosporin were obtained from Qualigens Fine Chemicals and GlaxoSmithKline Pharmaceuticals (Mumbai, India). All other chemicals used were of analytical grade and the solvents were redistilled before use.

**Determination of proteolytic activity, thermal stability, pH stability and nature of proteases**

Proteolytic activity of WTL (2.5-25 μg) was determined by the method of Murata et al., (1963), using fat free casein as substrate, as described in methods section of chapter 2. For thermal stability studies, 5 μg of WTL was subjected to heat treatment at 50, 70 and 100 °C for various time intervals (5, 10, 15 and 30 min) prior to the determination of proteolytic activity. To determine pH stability, WTL (5 μg) was pre-incubated in various buffers (citrate, acetate, phosphate and Tris-HCl) of pH ranging from 3.0 - 10.0 for 30 min, prior to the protease assay. Further, the nature of proteases was determined by pre-incubating WTL with specific protease inhibitors [PMSF (5 mM, serine), EDTA (5 mM, metalloprotease), pepstatin A (100 μM, aspartate) and IAA (100 μM, cysteine) in 1 ml reaction volume, for 30 min at 37 °C] prior to protease assay. The activity of WTL assayed in the absence of inhibitors was considered as 100%.

**Collagenolytic activity**

Varying concentrations of WTL (0-10 μg) was separately incubated with 50 μg each of type I and type IV collagen in a total reaction volume of 40 μl with Tris-HCl buffer (10 mM; pH 7.4) at 37 °C for 24 h. The reaction was terminated by adding 20 μl of reducing sample buffer (4% SDS, 6% β-mercaptoethanol and 1 M urea) and boiled for 7 min. 60 μl of this sample was loaded onto 7.5% SDS gel and electrophoresis was performed as previously described.
Wound healing activity

Wound healing activity of WTLP was determined using murine excision wound model as previously described in methods section of chapter 3. Various treatment groups included;

Group I: Wounds treated with saline (negative control)

Group II: Wounds treated with WTLP (10 mg/kg body wt.)

Group III: Wounds treated with heat inactivated (HD) WTLP (10 mg/kg body wt.) (Protease control)

Group IV: Wounds treated with WTLP pre-treated with PMSF

Group V: Wounds treated with Neosporin (Standard drug)

Group VI: Wounds treated with PMSF alone

The samples were applied over entire wound twice daily (10 mg/kg) for two groups of mice (n = 5) respectively for 9 days starting from day of wounding (day 1) and observed for wound contraction. Control groups were applied with saline (n = 5). Same concentrations of heat inactivated WTLP (HD-WTLP) was used as protease control. Studies were also performed using Neosporin (standard drug known to accelerated the healing of wounds), as positive control (n = 5). The experiments were also performed with proteases that were pre-incubated with PMSF and the inhibitor PMSF alone as a control (n = 7). For toxicity studies, PMSF solution (5 mM) alone was applied on the wound. Wound contraction (diameter of the wound calculated by tracing margins of the wound on a graph sheet) was monitored daily and the percentage of wound closure was determined by the relation,

\[
\text{Percentage wound closure} = \frac{\text{Initial area} - \text{nth day area} \times 100}{\text{Initial area}}
\]

Preparation of samples for biochemical and histological evaluation

Biochemical and histological analyses were carried out in the granulation tissues harvested from WTLP and all other treatment groups. The granulation tissues were excised on days 3, 6 and 9 following sample application. The granulation tissue
was de-fatted using acetone (Saha et al., 1994) and the collagen content was estimated. For MMP and catalase activities, the granulation tissues (100 mg) were homogenized in Tris-HCl (0.9% saline, 0.25%, Triton X-100, and 20 mM CaCl₂). The homogenate was centrifuged at 2,900 x g for 30 min and the supernatant was used for the assays. Along with treated tissues, equal weight of healthy skin was also processed identically for comparison. For histological examination, mice were euthanized and the healed portion of the skin was excised from representative control and various treatment groups. The skin tissues were fixed in Bouin’s solution followed by dehydration through different grades of alcohol and chloroform mixture. The processed tissue was embedded in paraffin and cut into 4 mm thick sections. The sections were stained with hematoxyline-eosin and were observed under Leitz Wetzlar (Germany type-307-148.002) microscope. The photomicrographs were taken using Photometrics Colorsnap CF camera (Roper Scientific Photometrics, Germany)

**Collagen content**

Collagen content of the healing skin tissues on days, 3, 6 and 9 following wounding was estimated in terms of hydroxyproline content according to the method of Neuman and Logan, (1950), as described in methods section of chapter 3.

**Catalase activity**

Catalase activity was determined by the method of Aebi (1984). Briefly, the reaction was initiated by adding varying amounts of tissue homogenate to 1 ml of 10 mM hydrogen peroxide and the reaction was monitored for 3 min and the change in absorbance was measured at 240 nm. Enzyme activities were calculated using 0.0394 mM⁻¹cm⁻¹ as absorption coefficient at 240 nm. Specific activity of catalase following HD-WTLP and WTLP treated samples were also calculated.

**MMP activity**

MMPs activity in the granulation tissue homogenates from WTLP treated and control mice were analyzed by gelatin zymography. Tissue homogenates (30 µg protein) were loaded onto a gelatin-incorporated polyacrylamide gel and subjected to electrophoresis. After electrophoretic run, the gel was processed as mentioned earlier.
Statistics

The results of the studies are expressed as the mean ± SD of minimum of five independent experiments performed in triplicates. Statistical analyses were carried out using Student’s t-test. The comparison between the groups were considered significant if p≤0.05.

Results

W. tinctoria latex supernatant contain thermostable proteases: The protein rich fraction of W. tinctoria latex extract was used as protease source (W. tinctoria latex proteases; WTLP). The fraction demonstrated caseinolytic activity in a dose dependent manner (Fig. 4.01a). Activity of WTLP at 10 μg protein concentration was 22 ± 1.1 U/h. In order to determine the thermostable nature, WTLP (5 µg/ml) was incubated at various temperatures (50, 70 and 100 °C) before determining proteolytic activity. The activity persisted even after boiling the samples at 70 °C for 30 min. However, the protease activity was completely abolished after boiling the samples for 30 min at 100 °C, demonstrating the thermo-stable nature of WTLP (Fig. 4.01b). Further, WTLP was found to be stable over wide range of pH (3.0 - 10.0) with maximum activity observed at pH 8.5 (data not shown).

WTLP demonstrate collagenolytic activity: The activity of WTLP on other protein substrates such as collagen (type-I and type-IV) was determined by SDS-PAGE analysis (Fig. 4.02). WTLP also hydrolyzed all 4 subunits of type I collagen resulting in the formation of small molecular weight protein and peptide fragments dose dependently (Fig. 4.02a). Similarly, WTLP also had potent proteolytic effect on α and β fragments of type IV collagen in a dose dependant manner forming smaller discrete molecular bands (Fig. 4.02b).

Topical application of WTLP augments excision wound healing in mice: The healing rate in WTLP treated group was significantly higher than (>2 fold) the control and HD-WTLP groups (49% vs. 18% and 20%; p<0.01) during initial 3 days respectively. Similarly, the percentage of wound closure in Neosporin treatment group was also significantly higher (38%, p<0.01) compared to control and HD-WTLP. The percentage of wound closure remained high in WTLP and was higher than Neosporin...
treatment group throughout the period of investigation (Fig. 4.03a and 4.03b). This data indicates the role of WTLP proteases in augmenting the wound healing process following topical application.

**Histological analysis confirms the accelerated wound healing effect of WTLP:** The skin tissues excised from the healed wounds on days 6 and 9 following topical application of the control, HD-WTLP, WTLP and Neosporin mice were subjected to histological analysis. Microscopic observation of the sections from healed tissues on day 6 post-wounding revealed the early granulation tissue containing numerous blood vessels in case of WTLP and Neosporin treated mice; in contrast HD-WTLP treated tissue consisted of poorly laid granulation tissue and lesser number of blood vessels. Further, tissue sections from day 9 demonstrated the recovery of skin structure and tissue continuity, with prominent regeneration of epidermal and dermal layers in mice applied with WTLP and Neosporin. On the contrary, less ordered skin structures were observed in HD-WTLP treated mice with less conspicuous dermal and epidermal layers (Fig. 4.03c).

**Pre-treatment of WTLP with PMSF purges the wound healing potential of WTLP:** To confirm, the role of WTLP serine proteases in wound healing process, we conducted similar wound healing experiments following pretreatment of WTLP with serine protease inhibitor PMSF. As depicted in Fig. 4.04a and 4.04b, PMSF-pretreated WTLP failed to enhance the wound contraction rate compared to control throughout the study period. Further, the contraction rate of wounds treated with PMSF alone was almost comparable to the control group, indicating that PMSF does not interfere with normal healing process. These findings further confirm the direct involvement of WTLP in enhanced wound healing activity.

**Biochemical analysis demonstrates high collagen content in WTLP treated granulation tissue:** Collagen content of the granulating tissues is an important biochemical parameter for evaluating the wound contraction rates (Fig. 4.05). Therefore, collagen content in terms of hydroxyproline in the granulating tissues from WTLP treated and control groups were determined at various time points. The hydroxyproline content in the WTLP and Neosporin treated groups was significantly higher than control groups on days 3 and 6 (Fig. 4.05a) (p<0.01). Further, on day 9
the hydroxyproline content in WTLP group was 1.5 fold (2444 ± 100 vs. 1579 ± 121μg/100 mg tissue) higher than the control respectively. When the hydroxyproline content of the granulation tissue was converted to collagen on days 3 and 6, the WTLP and Neosporin treatment groups remained significant (p<0.05) demonstrating the wound healing potential of topical application of WTLP (Fig. 4.05b).

WTLP treatment significantly enhance catalase and MMP activity: The variations in levels of antioxidant enzymes and MMPs provide vital evidence for the wound contraction rate. Therefore, the activities of catalase (Fig. 4.06a) and MMPs (Fig. 4.06b) were determined in the granulating tissues harvested from WTLP, Neosporin and control mice. Both the enzyme activities were significantly high in the treatment groups compared with control group on day 3, correlating with the enhanced wound contraction rate. Particularly, the catalase activity in WTLP group was 5.6 fold higher (16.7 ± 1.3 vs. 3 ± 0.3) than normal skin and HD-WTLP. Similarly the MMP activity in the tissue homogenate on day 3 was significantly higher in WTLP and Neosporin granulation tissues compared to other groups. These findings indicate the early onset of inflammatory phase, enabling the appropriate clearance of devitalized tissue and efficient scavenging of free radicals in the wound vicinity, facilitating the progression of healing towards repair phase. However, on days 6 and 9, no significant differences in the activities of these enzymes were observed, indicating the resolution of inflammatory phase, a pre-requisite of the healing process. These results further justify the direct involvement of WTLP in wound healing process and its application in traditional medicine.

Discussion

Plant latices have been used in Indian traditional medicine for treatment and management of different types of wounds including common wounds, cuts, burns, warts, ulcers and associated dermatological disorders. In addition, they possess properties such as anti-inflammatory, anti-microbial, larvicidal, insecticidal, analgesic activities (Thomas et al., 2008; Qian et al., 2010; Upadhyay, 2011). The medicinal properties of latices are attributed to the presence of biologically active components including secondary metabolites and hydrolytic enzymes which interfere with human/animal physiological pathways (Upadhyay, 2011). In latex, proteases
Topical application of serine proteases…..

contribute to the major portion of the hydrolytic enzymes and are significantly associated with pharmacological actions of latex (Rajesh et al., 2005). Similarly, latex from *W. tinctoria* is used to stop bleeding from fresh cuts and as wound healing agent (Kumar et al., 2007). Towards this line, our previous studies using crude and isolated proteases from latex have demonstrated the ability to promote blood clot formation and haemostatic activity (Rajesh et al., 2007). Based on the above evidences pertaining to clot inducing properties of latex, we aimed to determine the wound healing potential of WTLP in murine excision wound model and attempted to understand the underlying basic mechanisms.

Wound healing is an intricate and dynamic process, where proteases regulate crucial events such as hemostasis, fibrin plug formation, debridement, fibrin clot dissolution and tissue remodeling to facilitate and accelerate the healing process (Xue et al., 2006; Nguyen et al., 2009). Hemostasis is the initial and vital phase which involves the concomitant action of coagulation system and platelets, leading to fibrin plug formation. The hemostatic fibrin plug generated by the sequential proteolytic activation of blood clotting factors serves as provisional matrix and provides a platform that recruit cells and mediators of wound healing (Monaco and Lawrence, 2003; Diegelmann and Evans, 2004). Our results have already demonstrated the potential of latex proteases in inducing the fibrin clot formation on plasma suggesting a critical role starting at the initial phase of wound healing (Rajesh et al., 2005, 2006 and 2007). Further, the efficiency of wound healing depends on the appropriate clearance of necrotic tissue around wound site. This process termed debridement is facilitated by MMPs during the inflammatory phase of wound healing (Armstrong and Jude, 2002; Hayden et al., 2011). Our results indicate a significantly increased MMPs activity in the granulating tissues from mice treated with WTLP on day 3 suggest quick clearance of dead tissue or debridement. Proteases from Green bottle larvae (*Lucilia sericata*) are topically applied over chronic, non-healing wounds for clinical debridement (Chambers et al., 2003) where proteases hydrolyzed the major ECM proteins; collagen (type I and IV) and fibronectin by virtue of their ‘MMP-like’ activity. Similarly, WTLP exhibited gelatinolytic activity, confirming its action on ECM proteins. Furthermore, the WTLP efficiently hydrolyzed type I and type IV collagens, supporting the involvement in debridement process and supports its
Topical application of serine proteases...,

application on chronic, non-healing wounds for clinical debridement (Fig. 4.02a and 4.02b) similar to L. sericata proteases (Chambers et al., 2003). Therefore, our study demonstrates that WTLP is involved in removal of dead tissue from wound site, hence paves way for synthesis of new extracellular matrix proteins, augmenting tissue repair.

Fibrin clot formation is an important step in the initial stages of wound healing, but fibrin dissolution is a pre-requisite for tissue repair at later stages. This crucial event is facilitated by the endogenous protease, plasmin and allows laying of new tissue at the wound site by fibroblasts (Levi et al., 1999; Mehendale and Martin, 2001). In addition, dissolution of fibrin creates room for developing blood vessels which provide the area with nutrients and oxygen along with various immune cells ultimately resulting in tissue repair. Our previous observation of WTLP hydrolyzing washed whole blood as well as plasma clot (Rajesh et al., 2007) further provides confirming evidence behind the possible molecular mechanism leading to enhanced healing in WTLP treated wound compared to control group in the current investigation.

The physical evaluation of the wound contraction effect by WTLP (Fig. 4.03) was supported by histological studies which revealed the reappearance of skin structure with distinct layers of dermis and epidermis in the WTLP treated groups (Fig. 4.03c). The tissue in dermal region was more intact as compared to the control group, thus providing an evidence for the positive effect of WTLP towards wound healing in mice. Intactness of the tissue depends on formation, alignment and contraction of ECM molecules (Serini and Gabbiani, 1999). The size of wound and efficiency of reorganization of ECM molecules are vital in determining the extent of wound healing and reestablishment of tissue strength (Tomasek et al., 2002; Stroncek et al., 2009). The WTLP and Neosporin enhanced the contraction rate significantly (p≤0.01) compared to control group (Fig. 4.03). The presence of granulating tissue and abundance of blood vessels on day 6 skin section correlated with the wound contraction rate. The above observation was supported by the biochemical evaluation results demonstrating significantly high content of collagen in the granulation tissue (Fig. 4.05). The repair phase of wound healing is characterized by movement of fibroblasts to the wound site and synthesis and secretion of new ECM molecules
Topical application of serine proteases…..

(Duncan et al., 1999). Collagen, a major ECM protein is synthesized by fibroblasts, provides support for other ECM molecules (Bhogal and Bona, 2005). Collagen content in the granulation tissue increases with the progression of healing and hence is an important parameter to evaluate the healing process (Chithra et al., 1998). The increase in collagen content of granulation tissues from WTLP and Neosporin treated groups at day 3 was significant (p < 0.05), supporting the accelerated wound contraction rate in these cohorts. Moreover, WTLP enables early onset of repair phase supported by decrease in MMP activity on day 6 compared to day 3 (Fig. 4.06b). The MMP activities after 6 and 9 days are increased marginally, comparable to control group, indicative of the resolution of vital inflammatory phase and onset of repair phase, the re-establishment of the epithelium, restoring the tissue integrity.

Reactive oxygen species (ROS) generated by the phagocytic cells augment the inflammatory reactions during the process of healing (White and Heckler, 1990). The levels of ROS are elevated in wound region to eliminate the microbes in the vicinity of the wound and aggravate inflammatory process eventually preparing the wound site for repair phase (Mathieu et al., 2006; Woo et al., 2007). The levels of ROS subside with progression of healing. But, in chronic, non-healing wounds, the elevated ROS persists, causing cytotoxicity thereby hindering the process of healing (Chah et al., 2006). To eliminate ROS, expression of antioxidant enzymes such as catalase and superoxide dismutase is necessary. The level of catalase in the WTLP and Neosporin treated granulation tissue was substantially elevated on day 3 and subsided on day 6, indicating the normal expression pattern of the catalase similar to normal wound healing process. These findings indicate the beneficial auxiliary effect of WTLP towards wound healing.

In conclusion, *W. tinctoria* latex protease fraction promoted the wound healing activity and showed better wound contraction rate. The findings were supported by physical, histological and biochemical evaluations, providing biochemical validation for the extensive use of *W. tinctoria* latex in wound healing by folk medicinal practitioners. Further, the topical application of *W. tinctoria* latex proteases will be of immense value in clinical debridement of accumulated granulated tissue in chronic non-healing wounds as in case of diabetic patients and further will be an important strategy in wound management.
In spite of positive effects of proteases from plant latex towards wound healing, evaluation of toxicity is an important aspect of employing these agents to treat various types of wounds. Following careful assessment of toxicity, these proteases can be used in management of clinical conditions associated with abnormal clotting, bleeding and associated disorders.
Figures and tables

**Figure 4.01:** Proteolytic activity and thermal stability of WTLP.

**a.** Dose dependant proteolytic activity of WTLP (0 - 25 μg) was determined using fat free casein as substrate.

**b.** WTLP (5 μg) was incubated at 50, 70 and 100 °C for various time intervals (0 - 30 min) prior to the proteolytic assay. Results are expressed as mean ± SD (n = 6).
Fig. 4.02: Collagenolytic activity of WTLP.

a and b: Type-I (a) and Type-IV collagen (b) (50 μg each) were incubated separately with 0, 2, 4, 6, 8 and 10 μg of WTLP (lanes 1, 2, 3, 4, 5 and 6 respectively in both cases) in a total reaction volume of 40 μl of Tris-HCl buffer (0.01 M; pH 7.4) for 24 h. The reaction was terminated by adding denaturing buffer containing 4% SDS, 4% β-mercaptoethanol, 4 M urea and boiled for 7 min. The activity was visualized on 7.5% SDS-PAGE using Coomassie Brilliant Blue-R-250 staining.
Full thickness excision wound (10 mm diameter) was surgically made on the dorsal portion of mice. WTLP and HD-WTLP (150 μg) were applied twice daily on the wound (10 mg/ kg/day). The wound contraction was monitored by measuring the diameter of the wound using a graph sheet. The role of WTLP was confirmed by the topical application of HD-WTLP and Neosporin, a standard ointment was used as positive control.

a. Photographic monitoring of wound healing. Representative photographs of the wounds applied with the mentioned samples at day 0, 3, 6 and 9 post-wounding.

b. Percentage wound closure of WTLP treated wounds. Results are expressed as percentage wound closure (mean ± SD for n = 4, **= p<0.01).
Fig. 4.03 (Contd.): Excision wound healing activity of WTLP

c. **Histological examination of granulation tissue.** The representative healed portion of the skin from HD-WTLP, WTLP and Neosporin treated mice were excised on day 9 following wounding and the sections were stained with Hematoxyline and Eosin and photographs were taken using photometrics colorsnap CF camera.
**Fig. 4.04: Excision Wound healing activity of WTLP in presence of PMSF.**

WTLP pretreated with PMSF was applied twice daily on the wound as mentioned above. PMSF alone was applied to evaluate its toxicity and the results were compared with the control group. **a.** Representative photographs of the wounds applied with the above mentioned samples at day 0, 3, 6 and 9 post-wounding.  

**b.** Results are expressed as percentage wound closure (mean ± SD for n = 4).
**Fig. 4.05: Hydroxyproline and collagen content in granulation tissues.**

**a.** The granulation tissue from the control, WTLP and Neosporin treated mice was excised, hydrolyzed with 6 N HCl. 1 ml each of the sample was used for hydroxyproline estimation, the color developed was measured at 510 nm and the amount of hydroxyproline was determined using standard graph of hydroxyproline (0 - 10 μg).

**b.** The collagen content was calculated using the above results as explained in the methods section. Results are expressed as mean ± SD (n = 4). *= p<0.05, **= p<0.01.
Fig. 4.06: Catalase and MMP activity of granulation tissues.

**a.** The granulation tissue from the HD-WTLP, WTLP and Neosporin treated groups was excised and homogenized. Varying amounts of tissue homogenate was added to 10 mM Hydrogen peroxide and the reaction was monitored for 3 min at 240 nm. Results are expressed as mean ± SD (n = 4). *= p < 0.05, **= p < 0.01.

**b.** Equal concentration (30 μg) of tissue homogenate was loaded onto 7.5% SDS gel incorporated with 1% gelatin (HD-WTLP, WTLP and Neosporin in lanes 1, 2 and 3 respectively). Following electrophoresis, the gel was washed and kept in incubation buffer for 36 h at 37 °C. The activity bands were visualized after staining with Coomassie Brilliant Blue- R-250.