**Introduction**

Wounds are the inevitable events encountered during the lifetime of an individual resulting from mechanical, chemical or surgical damage, microbial infection or an underlying pathological condition (Ramzi et al., 1994). The host aptly responds to a wound through a series of events as a damage control response to restore the tissue integrity and function (Crovetti et al., 2004). This complex physiological response, termed wound healing is controlled and coordinated by immune cells, extracellular matrix (ECM) and components of hemostatic system (Stadelmann et al., 1998). In spite of the efficient host response, some pathophysiological conditions/infections result in altered healing response, in most cases ending up with delayed healing or non-healing chronic wounds (Singer and Clark, 1999). To overcome/minimize the complications leading to altered healing, wound care and management is the remedy. Conventionally, the strategies of wound care involve the administration of synthetic antibiotics and anti-inflammatory agents, with or without debridement; in extreme cases grafting is the last resort (Cowan et al., 2011). But, these are associated with undesirable effects, including side effects, pain, discoloration of skin and complications of histocompatibility and tissue rejection (Wysocki and Dorsett-Martin, 2008). These adverse effects need to be carefully addressed and overcome by alternate/complementary medicinal strategies. In this regard, herbal medicines would be suitable agents for wound care, since they provide suitable conditions to augment physiological healing process by offering minimal adverse effects (Purna and Babu, 2000). Latex, an important plant based component is widely used for topical application on various types of wounds including common wounds, cuts, ulcers, burns, boils, cracks and warts (Ayyanar and Ignacimuthu, 2009). Although extensively used, very few reports explain the detailed molecular mechanisms underlying the wound healing property of latex. The ability of plant latex to facilitate wound healing process is attributed partly to the secondary metabolites and mostly to proteolytic enzymes contained in it (Rajesh et al., 2005; Upadhyay, 2011). Proteolytic enzymes from plant latices have been reported to promote healing of wounds in experimental animals (Badgujar and Mahajan, 2011). Further, papain and chymopapain, from *Carica papaya* latex are clinically approved wound care agents (Udod and Storozhuk, 1979). Most of the proteases reported to facilitate
wound healing by hemostasis and debridement process, where necrotic tissue is cleared from the wound site. Whereas, molecular mechanisms of other proteases still remain unclear. Reports from Rajesh et al., (2007) and Shivaprasad et al., (2009) indicate that plant latex proteases possess exhibit clot inducing and clot hydrolyzing properties. Clot formation is vital for hemostasis, the initial phase of wound healing, whereas clot hydrolysis is a pre-requisite for the events of regenerative phase (Clark, 2011). Based on the available reports, the present study aims at evaluating the wound healing potential of plant latex proteases. Further, a comparative account on the underlying molecular mechanisms by which latex proteases augment the natural healing response will be established, which will substantiate the role of latex and latex proteases as wound healing agents.

Materials and Methods

Latex yielding plants and processing of latex

After initial screening, *W. tinctoria* (Apocyanaceae), *S. grantii* (Euphorbiaceae), *C. gigantea* and *P. extensa* (Asclepiadaceae), were selected for the study. The plants were identified and authenticated by Dr. G. Sharanappa, Associate Professor, Department of Studies in Bioscience, University of Mysore, Hassan, Karnataka, India. Processing of latex was carried out as mentioned in methods section of Chapter 2. The protein content of this enzyme fraction was estimated according to the method of Lowry et al., (1951). The protein rich fractions from latex of *W. tinctoria*, *S. grantii*, *C. gigantea* and *P. extensa* were used as protease source and are abbreviated as WTLP, SGLP, CGLP and PELP respectively.

Human Plasma

Platelet poor plasma for experiments was prepared as described in methods section of chapter 2.

Chemicals

Ketamine and xylocaine were purchased with the prescription of University Medical Practitioner. Gelatin, trypsin and pepstatin A (microbial source) was obtained from Sigma Chemicals (St. Louis, MO, USA). Papain, PMSF, IAA, EDTA and para-dimethyl amino benzaldehyde (p-DMAB) were purchased from Sisco Research
Laboratory, Mumbai, India. Neosporin was purchased from GlaxoSmithKline Pharmaceuticals, Mumbai, India. All the other chemicals used were of highest analytical grade. Solvents were distilled before use.

**Animals**

Adult Swiss Albino mice (30-35 g; either gender) were obtained from The Central Animal House Facility, University of Mysore, Mysore. The animals were maintained in polypropylene cages with 12 h light dark cycle. The animal care and handling were conducted in compliance with the Regulations for Animal Research. The animal experiments were carried after reviewing the protocols received from The Animal Ethical Committee of the University of Mysore, Mysore (Sanction order No. MGZ/2518(b)/2009-10).

**Proteolytic activity**

Proteolytic activity was carried out according to the method of Murata et al., (1963), as described in methods section of chapter 2.

**Gelatinolytic activity**

Gelatinolytic activity by zymogram assay was carried out according to the method of Heussen and Dowdle (1980), with slight modifications. 1.5% gelatin was incorporated into 10% SDS resolving gel. 10 μg each of latex protein fractions, trypsin and papain were loaded onto the gel and electrophoresis was carried out at a constant voltage of 100 V at room temperature. After electrophoresis, the gel was washed with Triton X-100 (2.5, 1.25 and 0.5%) and subsequently with distilled water to remove SDS. The gel was incubated with 50 mM Tris-HCl buffer pH 7.6, containing 10 mM CaCl₂ and 150 mM NaCl at 37 °C for 24 h and the gel was stained with Coomassie Brilliant Blue R-250 stain. Clear zones against dark blue background indicated the gelatinolytic activity.

**Human plasma clot hydrolyzing activity**

Human plasma clot hydrolyzing activity was carried out according to the method of Rajesh et al., (2005). Plasma (100 μl) was mixed with 20 μl of Tris-HCl buffer (10 mM; pH 7.4) and 30 μl of 250 mM CaCl₂ and allowed at 37 °C for 2 h for
clot formation. The plasma clot was washed thoroughly with phosphate buffered saline (PBS) to remove interfering plasma proteins. Washed fibrin clot was incubated with different concentrations of WTLP, SGLP, CGLP, PELP, trypsin and papain in a reaction mixture of 40 µl Tris-HCl (10 mM; pH 7.4) for 2 h at 37 °C. The reaction was terminated by adding 20 µl of sample buffer containing 4% SDS, 6% β-mercaptoethanol and 6 M urea, boiled for 6 min and centrifuged at 1500 x g for 10 min. An aliquot of 25 µl was loaded on to 10% SDS gel and the electrophoresis was carried out under reducing condition. After electrophoresis, the gel was stained using 0.25% Coomassie Brilliant Blue R-250 (methanol : water : acetic acid :: 5 : 4 : 1) and destained using methanol : water : acetic acid :: 5 : 4 : 1. The change in the banding pattern of the protease treated clot was compared to that of control, where clot was incubated with Tris-HCl (10 mM; pH 7.4) alone.

**Fibrinogenolytic activity of latex proteases**

Fibrinogenolytic activity was carried out according to the method of Ouyang and Teng (1976), as described by Rajesh et al., (2005). Briefly, 50 µg of human fibrinogen (in 10 mM Tris-HCl; pH 7.4) was incubated with varying concentrations of latex proteases, trypsin and papain for 2 h at 37 °C. The reaction was terminated by adding 20 µl of sample buffer containing 4% SDS, 6% β-mercaptoethanol and 6 M urea, boiled for 6 min and centrifuged at 1,500 x g for 10 min. The reaction mixture was loaded on to 10% SDS gel and the electrophoresis was carried out under reducing condition. The activity was visualized by Coomassie Brilliant blue staining. The appearance of low molecular weight degraded bands indicated the fibrinogenolytic activity in comparison to the intact sub-units of fibrinogen.

**Wound healing activity**

Wound healing activity was carried out using excision wound model in mice according to the method of Frank and Kämpfer (2003). Mice were anaesthetized by intraperitoneal injection of ketamine (100 mg/kg body wt.) and xylocaine (10mg/kg body wt.) combination. The hair on the dorsal surface was removed and a full thickness excision wound of 1 cm diameter was made using sharp scissor. Experimental groups consisted of topical application of; saline, crude latex, dewaxed latex (undialyzed fraction), latex protease fractions, heat denatured protease fractions,
Comparative account on wound healing..., inhibitory pre-treated proteases, reference proteases (trypsin and papain), standard drug (Neosporin) and inhibitor alone. Protein fractions (150 µg; 10 mg/kg body wt.) from different plant latices were applied twice daily on the wound for 12 days. The changes were recorded (diameter of the wound calculated by tracing the margins of the wound on a graph sheet) daily. For protease control, heat denatured proteases (HD-WTLP, HD-SGLP, HD-CGLP and HD-PELP) were applied on wounds. Trypsin and papain were used as reference proteases and Neosporin was used as standard ointment. Inhibitor pre-treated proteases were applied on wounds. Inhibitors alone were applied to determine their effect on physiological wound healing. Wounds which were treated with saline were used as negative control. The percentage of wound closure is determined by the relation,

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

**Collagen content**

Collagen content in the healed skin tissues was estimated according to the method of Neuman and Logan (1950), in terms of the amount of hydroxyproline present in the skin tissues. The granulation tissue from different treatment groups was defatted using acetone and hydrolyzed with 6 N HCl for 10 h. To 0.5 ml of de-fatted and HCl hydrolyzed granulated tissue, 1ml each of 10 mM copper sulphate, 2.5 M sodium hydroxide and 6% hydrogen peroxide were added in succession and mixed thoroughly. The reaction contents were kept in boiling water bath at 80 °C for 5 min with constant shaking. The tubes were cooled by immediately transferring the tubes on ice and to this 4 ml of 3 N sulphuric acid and 2.5 ml of 5% p-DMAB (in n-propanol) were added and mixed thoroughly. The tubes were heated in water bath at 70 °C till the development of pink color and cooled to room temperature. The color developed was measured at 530 nm. The amount of hydroxyproline was calculated using the standard graph of hydroxyproline (0-20 µg). Hydroxyproline concentration obtained was correlated to the collagen content in the healed portion of skin using the relation,

\[
\text{Collagen content} = \frac{\text{Amount of hydroxyproline in the sample} \times 7.45}{\text{Amount of sample}}
\]
**Statistics**

The results of biochemical and pharmacological experiments were expressed as the mean ± S.E.M. of three independent experiments. ANOVA was performed for multiple comparisons. Means were compared using Student’s *t*-test at 5% level of significance.

**Results**

**Processing of latex yielded protein rich fraction:** Latex from the selected plants was separately processed to get the protein rich fraction after the removal of wax and micro constituents such as secondary metabolites and low molecular mass peptides. The protein rich fraction was used as protease source for further experiments.

**Latex protein fractions hydrolyzed casein in concentration dependent manner:** Protein rich fractions from plant latices showed dose dependent activity response and exhibited moderate to high specific activities towards casein. Latex proteases belong to either serine or cysteine super family, as determined by inhibition studies using specific protease inhibitors (Chapter 2).

**Latex protease fractions showed gelatinolytic (MMP-like) activity:** MMP-like activity of latex proteases was evaluated by substrate gel assay; using gelatin coated SDS polyacrylamide gel. Latex protein fractions efficiently hydrolyzed gelatin, evident by the clear zones of hydrolysis against the dark blue background. In case of latex proteases, activity bands were not observed, because SDS resistant nature of latex proteases. The gelatinolytic activity of latex proteases is indicative of their action towards ECM proteins, similar to matrix metalloproteases, which mediate vital events of wound healing. Similar results were obtained with the reference proteases used – trypsin and papain (Fig. 3.01).

**Latex protease fractions hydrolyzed fibrinogen:** The action of latex proteases was further tested on fibrinogen and fibrin by observing the hydrolysis pattern of the respective substrates on SDS polyacrylamide gel. The hydrolytic action of latex proteases was concentration dependent and after 2 h of incubation, all the sub-units of fibrinogen (Aα, Bβ and γ chains) and fibrin (α-polymer, γ-γ dimer, α-chain and β-chain) were susceptible for hydrolysis by latex proteases. Similarly, non-specific
hydrolysis was observed in case of papain. In contrast, trypsin showed specificity towards Aα and Bβ chains of fibrinogen. The γ chain of fibrinogen was resistant to the action of SGLP and trypsin. In case of fibrin, α-polymer and γ-γ dimer were resistant to the action of trypsin. The action of latex proteases and standard proteases towards respective protein substrates was distinct as evidenced by the characteristic low molecular mass fragments generated by hydrolysis of respective proteins (Fig. 3.02 and 3.03)

**Proteases from plant latices augment the wound healing process:** Wound healing potential of latex proteases was determined by murine excision wound model. Proteases from plant latices were applied topically on the wound and healing activity was monitored daily. The extent of healing in latex protease treated groups (97.80±3.7%, 97.11±2.9%, 96.50±3.1%, 91.79±2.56% respectively for WTLP, PELP, CGLP and SGLP) was higher in comparison to the control group in which the wounds were untreated (82.61±3.0%). Wound contraction in reference proteases – papain and trypsin treated groups was 95.58±6.2% and 90.17±3.5% respectively. Wound contraction was significantly higher during initial 3 days of latex protease application, with more than 2 fold increased contraction (p<0.005). In similar lines, wound contraction during initial 3 days of topical application of trypsin and papain was 1.5 fold more in comparison to control group. In contrast, Neosporin treated group did not show significant healing during initial 3 days, but showed better healing activity on 12th day of treatment, compared to saline treated wound (Fig. 3.04 and Table 3.01). The wound contraction rate in wounds treated with crude latex, dewaxed latex, heat denatured latex protease fractions, inhibitor pre-treated protease fractions and wounds treated with inhibitor alone did not show significant healing and was comparable to saline treated group. In addition, an important observation was the allergic and burn like appearance following topical application of *P. extensa* latex (crude and dewaxed extract) - data not shown.

**Enhanced collagen content was observed in protease treated granulation tissue:** Collagen content in the granulation tissue is an important biochemical parameter to monitor the extent of healing. Processed granulation tissue from protease treated groups showed higher collagen content [8.9% in WTLP, CGLP and PELP treated groups (p<0.05); 4.4, 6.6 and 6.6% respectively in SGLP, trypsin and Neosporin]
Comparative account on wound healing....,

treated groups (p>0.05)] compared to the control group (Fig. 3.05a). In all the
treatment groups, significant increase in collagen content was observed during initial
3 days of topical application (data not shown). The findings of collagen content are in
agreement with the wound contraction rates. The increase in granulation tissue
collagen content in treatment groups normalized with control groups is depicted.
Collagen content in all the groups are compared with that of normal (uninjured) skin
(Fig. 3.05b)

Discussion

Latex, being an important plant derived component plays a vital role in plant
physiological processes. It regulates water balance, storage of nutrients and is a
defensive weapon against invading herbivorous insects and plant pathogens (Agrawal
and Konno, 2009). Apart from this, latex exhibits various pharmacological effects and
is widely used in folk medicine to stop bleeding on fresh cuts and for healing of
wounds (Thankamma, 2003). Latex is a rich source of proteases, which are
responsible for the observed pharmacological effects (Rajesh et al., 2005). The
inhibition studies using specific protease inhibitors revealed the presence of serine
proteases in Apocyanaceae and Euphorbiaceae members and cysteine proteases in
Asclepiadaceae members. In support of these findings, reports have shown that any
given plant family contains only one type of protease and could be used as a possible
chemotaxonomic tool for classification of plants (Shivaprasad et al., 2009).

Wound healing is an intricate, dynamic process regulated by many enzymatic
and non-enzymatic mediators. Among enzymatic mediators, proteases play vital role
in all the four phases of wound healing. Along with the endogenous proteases, topical
application of proteases from different sources facilitates the process of wound
healing. The interference of latex proteases in hemostasis and fibrinolysis is an
important evidence for their involvement in wound healing (Shivaprasad et al., 2010).

Wounds, irrespective of the cause, results in the tissue damage and the
damaged components of the tissue (devitalized tissue) remain at the wound site. The
efficiency of wound healing depends on the appropriate clearance of dead, necrotic
tissue from the wound site (Stroncek et al., 2009). Removal of dead tissue also
ensures that microbial colonization in the wound site is reduced. This process termed
as debridement is done by matrix metalloproteases (MMPs) during the inflammatory phase (Moali and Hulmes, 2009). Activity of MMPs is routinely evaluated by gelatinolytic activity. In similar lines, gelatinolytic activity of latex proteases was evaluated by clear zones of hydrolysis against blue background. Gelatinolytic activity is attributed to the proteases acting on ECM proteins; gelatin hydrolyzing proteases, such as MMPs play vital roles in normal physiological responses including wound healing. The primary role of MMPs include the clearance of non-viable tissue at the wound site, ensuring the completion of decontamination process, decreasing the risk of microbial infection, in addition to enabling the onset of proliferation phase and sequence of events leading to tissue repair fractions suggests that they facilitate the clearance of dead tissue (Armstrong and Jude, 2002; Hayden et al., 2011). This action of latex proteases supports the action of endogenous MMPs in evacuating the dead tissue. In addition to reducing the microbial load at the wound site, the process of removal of dead tissue from wound site paves way for synthesis of new extracellular matrix proteins, enabling the onset of events of tissue repair. Thus, ‘MMP-like’ activity of latex proteases will be a vital mechanism for the augmentation of healing process and minimizing the risk of infection (Stroncek et al., 2009).

Proteolytic enzymes from plant latex, irrespective of the plant family and nature of proteases exhibit procoagulant effect and facilitate the formation of clot, thereby limiting the loss of blood and reducing the risk of microbial contamination. The information regarding the mechanisms of latex proteases in exhibiting procoagulant effect is limited, with very few reports indicating the site specific actions towards blood coagulation factors (Devaraj et al., 2009; Shivaprasad et al., 2010). Reports have suggested the action of plant latex proteases towards fibrinogen, the coagulation factor in circulation. Proteases may have direct action on fibrinogen or may activate the coagulation factors which eventually lead to the formation of thrombin, which cleaves fibrinogen. Fibrinogenolytic proteases have been reported from plant latex. However, these proteases lack specificity towards sub-units of fibrinogen and hydrolyze all the sub-units, unlike snake venom proteases which are specific for Aα and Bβ chains of fibrinogen (Swenson and Markland, 2005, Rajesh et al., 2006).
Proteolytic enzymes from latex, apart from induction of clot, exhibit clot hydrolyzing activity, a vital process during the repair phase of healing. Although fibrin clot supports initial events of wound healing, appropriate breakdown and clearance of clot is a pre-requisite for the events of repair phase, especially, angiogenesis to provide oxygen and nutrition for regenerating tissues and synthesis of new ECM (Clark, 2001). The process of clot dissolution is mediated by plasmin, leading to the events of tissue repair and is also involved in tissue remodeling (Green et al., 2008). In addition to endogenous action of plasmin, topical application of fibrin hydrolyzing enzymes from plant, animal and microbial source will be an important strategy for promotion of wound healing. This property of latex proteases would be an important contribution for the enhanced wound healing of the protease treated wound compared to the control group.

The wound contraction rate gives an indication of the extent of healing. During the remodeling phase, ECM molecules undergo contraction and alignment to reduce the wound size and to re-establish tissue strength (Stroncek et al., 2009). The size of the wound and the efficiency of reorganization of ECM molecules are vital factors determining the extent of wound healing. The latex proteases as well as reference proteases enhanced the contraction rate in comparison to the control group, indicating the positive role of proteases towards wound healing. In support of these findings, the increased collagen content of the protease treated groups substantiates the enhanced wound contraction. Collagen content in the granulation tissue is an important biochemical parameter to evaluate the extent of healing. The collagen content increases with the progression of healing and is directly proportional to the wound contraction rate (Chithra, 1998).

In conclusion, the results indicate positive effect of latex proteases towards wound healing, evaluated by the extent of wound contraction and determination of collagen content in the granulation tissues of protease treated groups. In addition, the possible mechanisms of action of latex proteases in promoting the healing of wounds have been established. In future, the purification, characterization and molecular mechanisms of the purified protease(s) will further strengthen the available evidence for the use of latex as WOUND HEALING agent. Further, the role of exogenously applied latex proteases in tissue remodeling needs to be evaluated. Also, the topical
application of latex proteases might be useful for clinical debridement of granulated tissue in case of chronic non healing wounds.

Based on the preliminary data, non-toxic nature and the ethnopharmacological usage to treat variety of skin disorders and ulcers, *W. tinctoria* latex was selected for the detailed study using excision wound model in mice.
Figures and tables

Fig. 3.01: Gelatinolytic activity of latex proteases.

Latex protease fractions were loaded on to 12.5% SDS impregnated with 1.5% gelatin and electrophoresis was carried out under non-reducing conditions. After electrophoresis, the gel was washed with trition-X-100 and water. The gel was kept in incubation buffer for 24 h at 37 °C. The activity bands were visualized after Coomassie staining. Lanes 1-6: 2 µg of WTLP, 10 µg each of SGLP, trypsin, CGLP, PELP and papain respectively.
Fig. 3.02: Fibrinogenolytic activity of latex proteases.

Human fibrinogen (50 μg) was separately incubated with latex protease fractions at 37 °C for 2 h. The reaction was terminated by adding 20 μl of stopping buffer, boiled for 6 min. The reaction contents are loaded on to 10% SDS gel and the electrophoresis was carried out at constant voltage. Lane 1: Clot + buffer; 2 to 6 – Clot incubated with 2 μg WTL, 10 μg each of SGLP, trypsin, CGLP, 2 μg of PELP respectively.
Fig. 3.03: Fibrinolytic activity of latex proteases.

Washed plasma clot (fibrin) was separately incubated with latex protease fractions at 37 °C for 2 h. The reaction was terminated by adding 20 µl of stopping buffer, boiled for 6 min and centrifuged at 1,500 x g for 10 min. An aliquot of 25 µl was loaded on to 10% SDS gel and the electrophoresis was carried out at constant voltage. Lane 1: Clot + buffer; 2 to 7 – Clot incubated with 2 µg WTL, 10 µg each of SGLP, trypsin, CGLP, 2 µg of PELP and 10 µg of papain respectively.
Fig. 3.04: Excisional wound healing activity of plant latex proteases.

Circular excision wound (10 mm diameter) was surgically created on the dorsal side of mice. Latex protease samples were topically applied on the wound twice daily (10 mg/kg body wt.). The progression of healing was monitored by measuring the wound area. Results are expressed as percentage of wound closure (mean ± S.D; n=5).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 3</th>
<th>Day 6</th>
<th>Day 9</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.91±1.1</td>
<td>55.12±1.9</td>
<td>72.83±2.4</td>
<td>82.61±3.0</td>
</tr>
<tr>
<td>WTLP</td>
<td>49.04±2.34**</td>
<td>82.27±3.2*</td>
<td>89.33±3.2*</td>
<td>97.80±3.7*</td>
</tr>
<tr>
<td>SGLP</td>
<td>45.06±1.9**</td>
<td>67.69±2.7*</td>
<td>81.53±3.3</td>
<td>91.79±2.56</td>
</tr>
<tr>
<td>Trypsin</td>
<td>38.46±2.1</td>
<td>58.97±2.9</td>
<td>78.56±2.9</td>
<td>90.17±3.5</td>
</tr>
<tr>
<td>CGLP</td>
<td>50.56±1.9**</td>
<td>65.76±2.4*</td>
<td>81.80±3.7</td>
<td>96.50±3.1*</td>
</tr>
<tr>
<td>PELP</td>
<td>52.46±1.89**</td>
<td>73.07±2.1*</td>
<td>90.38±2.5*</td>
<td>97.11±2.9*</td>
</tr>
<tr>
<td>Papain</td>
<td>30.06±4.2</td>
<td>61.71±3.6</td>
<td>83.13±5.6</td>
<td>95.58±6.2*</td>
</tr>
<tr>
<td>Neosporin</td>
<td>26.66±1.0</td>
<td>40.74±2.6</td>
<td>79.66±2.5</td>
<td>89.65±3.0</td>
</tr>
</tbody>
</table>

**- p≤0.005 (extremely significant); *- p≤0.05 (significant) in comparison to control
Fig. 3.05a: Collagen content in granulation tissue of various treatment groups (12th day).

Granulation tissues from different treatment groups were harvested on 12th day of treatment. The tissues were processed and collagen content was determined as mentioned in the methods section. Results are expressed as mg of collagen/100mg tissue. * - p≤0.05.

Fig. 3.05b: Percentage of granulation tissue collagen normalized with control.

The increase in collagen content of granulation tissues are depicted after subtracting the collagen content of granulation tissues in treatment groups with that of control.