PHARMACOLOGICAL PROPERTIES OF A PROTEASE FROM FICUS HISPIDA LINN

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ABSTRACT: A sulphydryl plant protease present in the latex of Ficus hispida Linn affects hematological values in mice. The isolated protease was found to increase clotting time and erythrocyte sedimentation rate while haemoglobin content, RBC count and WBC count were decreased in a dose dependent manner. Ointment containing 1.0% (w/w) hispidain in washable ointment base showed good wound healing property in mice. The protease also possesses mild anti inflammatory activity.

Ficus hispida Linn (Family: Moraceae), is a moderate sized tree available in India and some other tropical countries¹-⁵. Apart from other uses, different parts of the plant are used in the treatment of haemorrhage of nose and mouth, some diseases of blood, and treatment of boils and wounds in the traditional system of medicine²,³. The plant on injury exudes a milky and sticky latex⁴. The isolation of a protease form the latex, its purification and some of its physico-chemical properties have been reported⁶. The temperature and pH for optimum activity was 40ocand 7.0 respectively using casein as substrate. Later, we purified the protease further by sephadex G-200 gel filtration upto 36 folds with a yield of 5.22% and determined the molecular weight of the protease by sephadex G-100 gel filtration which was found to be around 23,700 daltons (unpublished work). Acute toxicity of the sample of protease has been studied (LD₅₀=1230 mg/kg body weight in mice, intraperitoneal).

The present work deals with the study of the effects of the protease on hematological values in mice, on healing of wounds, and its effect on in inflammatory conditions.

EXPERIMENTAL

Materials:

The protease isolated from the crude extract of the latex of Ficus hispida Linn by precipitation with 40-60% ammonium sulphate followed by dialysis and iyophilization⁶ was used for the study. Albino mice of either sex weighing between 18-24 and albino rats of either sex weighing between 150-200g where purchased from M/S B.N Ghosh, Calcutta. Heparin Sodium injection (5000 i.u. per ml, Beparine, Biological E.), Soframycin skin cream containing 1.0% (W/W) Framycetin Sulphate in; washable base (Hoechst Marion Roussel itd., Mumbai) and Vovaran injection (Dominion pharmaceuticals pvt. Ltd., Bangalore) were purchased from market. Water washable ointment base⁷ was prepared in our laboratory. All other chemicals were purchased form either E. Merck India Ltd., or Ranbaxy laboratories Ltd and were of analytical grade.

Methods:
Effect of the protease on haematological values in mice: Albino mice fed with standard pellet diet and tap water ad libitum were kept in identical laboratory conditions for five days. The animals were divided into four groups, each group containing ten mice. Protease solution prepared in distilled water was injected intraperitoneally and daily for 15 days at the dose level of 136 mg/kg (low), 205 mg/kg (moderate) and 410 mg/kg bodyweight (High) to the first, second and third group of mice respectively. The fourth group received water (5.0 ml/kg) daily. Animals were sacrificed 24 hours after the last dose. Blood was collected from tailveins and by cardiac puncture with the help of syringe previously rinsed with heparin.

Estimation of red blood cell count, white blood cell count, differential count of leucocytes, haemoglobin content of whole blood erythrocyte sedimentation rate (ESR) were carried out according to the normal procedure for determination of these parameters. For estimation of clotting time, blood from tail was allowed to fill capillary tube held horizontally. After few seconds, sections of the tube were broken off once every ten seconds till the appearance of fine fibrin threads at the broken end indicating clotting of the broken end indicating clotting of the blood. The time required from appearance of the blood at the tail to the time of clotting measured by stop watch was the clotting time of the blood.

Wound healing responses of the protease:

Mice maintained on standard pellet diet with tap water ad libitum were acclimatized for a period of 15 days. The hairs from the dorsal plane of thora columbar region of each animal was removed by shaving and rectangular wound of about 1.5 cm x 0.5 cm was produced surgically extending to the muscle to a depth of about 0.2 cm. The animals were divided randomly into five groups (Groups 1 through 5) containing five mice in each group. Animals of group 1 were left as untreated control while the treatment of wounds of the mice of groups 2,3,4 and 5 were started immediately by the application of ointment once a day as follows:

- **Group 1:** Untreated control.
- **Group 2:** Hydrophilic ointment base.
- **Group 3:** Soframycin skin cream.
- **Group 4:** Ointment containing the protease (0.5% w/w) in hydrophilic ointment base.
- **Group 5:** Ointment Containing the protease (1.0%w/w) in hydrophilic ointment base.

On 5th day from the creation of wound, the tissue extending about 0.2 cm beyond the wound area was collected from one mouse of each group. Similarly, the wound tissue from one mouse of each group was collected on 10th, 15th and 20th day and were processed according to laboratory procedure. The histopathological changes during the healing process were studied.

Anti-inflammatory activity of the protease: The anti-inflammatory activity of the protease sample was evaluated by carragenan induced rat hind paw oedema method. The animals were acclimatized for a period of seven days maintaining on standard pellet diet and were kept in fasting condition overnight before starting the experiment. The rats were divided into three groups taking five animals in each group.

Protease solution, 25.0 mg/ml and carragenan solution, 1.0% (w/v) were prepared in normal saline. The animals of prepared in normal saline. The animals of group I (Control) received saline (1.0 ml kg), group II (Standard) received diclofenac
sodium (25.0 mg/kg) and group III (Test) received protease sample (25.0 mg/kg) in right hind paw. After one hour of this treatment 0.1 ml of 1.0% carragenan solution was injected to the same paw of each animal of all the groups. All the injections were given by sub plantar route of administration. Observation in mercury plethysmometer were made immediately after carragenan injection (Zero hour) and repeated after every one hour for three hours. The precent swelling of the paw of each animal at different times were calculated and the average paw swelling of the rats of groups II and III were compared with that of control rats (Group 1) and the percent inhibition of oedema formation was determined.

RESULTS AND DISCUSSION

The results of the haematological studies have been summarized in Table -1 and Table -2 and in Fig.1, Fig 2, Fig 3. An increase in the clotting time and erythrocyte sedimentation rate (ESR) were observed in all the three dose leaves. The increase in clotting time was 15.4% with moderate dose and 16.4% with high dose. In low dose, the increase was insignificant. The increase in clotting time might because of proteolytic action of the enzyme sample on protein factors responsible for blood clotting. The increase in ESR was increased by 12.4%, 53.9% and with the three dose level respectively. This increase in ESR is possible due to decrease in the level of albumin or nucleoprotein 13 as a consequence of proteolytic action of the enzyme sample. Haemoglobin content, RBC count and WBC count decreased in a dose dependent manner. Haemoglobin content was decreased by 10.8%, 15.9% and 26.4% respectively in low, moderate and high dose treated mice. The RBC count was decreased by 7%, 10.3% and 13.9% respectively whereas WBC count was decreased by 6.9%, 9.3% and 10.1% respectively for the three dose levels. The decrease in RBC count might be due to loss of some stimulus that constantly act on the bone marrow in order to replace the lost cells, Haemoglobin content is related with red cell count and its decrease is because of decrease in the red cell count in protease treated animals. The rise in neutrophil and fall in lymphocyte count might be due to increased hydrolysis of nucleoprotein because of protease action. Thus it clearly signifies that the protease affects haemopoietic system and greatly alters the level of haematolgical parameters in animals.

The observations on wound healing responses of the protease are shown in tabular form in Table – 3. The untreated control wounds and the wounds treated with washable ointment base showed delayed and incomplete healing even on the twentieth day while the wounds treated with soframyacin skin cream and ointment containing 0.5% (w/W) protease showed a better healing quality than the first two groups and were found to be equally effective on wound healing. The 1.0% (w/w) protease showed a better healing quality than the first two groups and were found to be equally effective on wound healing. The 1.0% (W/W) protease ointment, on the other hand revealed a much faster healing with normal epithelialization and keratinization on 20th day. Microphotographs showing the wound healing response of the protease have been given in Fig, 4, 5 and 6. The use of proteolytic enzymes in the treatment of wound the been indicated earlier also.14,15 Nath and Dutta16 have reported the wound healing property of curcain, a protease isolated form Jatropha curcas Linn. The selective digestion of the dead tissue and
cells of the wound is achieved by the proteolytic enzymes which facilitate wound drainage decreasing the time needed for healing.

The results of the study on anti-inflammatory activity are presented in Table - 4. The percent swelling of the paw of rats after one, two and three hours of carrageen injection were calculated with respect to the observation at Zero time using the equation

\[
\text{Percent Swelling} = \frac{V-V_i}{V_i} \times 100
\]

Percent inhibition = \[\text{Percent swelling of enzyme treated group} \times 100 \]

\[
\text{Percent swelling of control group}
\]

Percent inhibition of oedema formation by the protease was remarkably lower than that of the diclofenac sodium in all the observations of respective intervals. The inhibition of oedema formation by the protease was found highest at one hour after carragenan injection with gradual decrease with time. Several proteolytic enzyme preparations are used to reduce acute inflammations following surgery or athletic injury. Inhibition of swelling in carrageen induced inflammation by the protease might be due to disruption of one or more enzyme systems which are responsible for genesis of inflammation. Of course, the anti-inflammatory action was notably weaker than that of the established anti-inflammatory drug.

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**TABLE 1: Effect of the protease form Ficus hispida on Haematological parameters in mice.**
(Values are mean ± SEM of 10 experiments in each case)

| Parameters     | Water 5.0ml/kg b.w | Protease Solution |
|----------------|--------------------|-------------------|
| Clotting time (sec) |                    |                   |
| 216.5 ± 4.54    | 227.6 ± 4.05       | 249.9 ± 2.06*     |
| 252.0 ± 1.69*   |                    |                   |
| Haemoglobin content (%) |                |                   |
| 76.0 ± 1.09     | 67.8 ± 0.84*       | 63.9 ± 0.82*      |
| 55.9 ± 0.82*    |                    |                   |
| ESR (mm/hr)     |                    |                   |
| 0.89 ± 0.03     | 1.0 ± 0.03*        | 1.37 ± 0.07*      |
| 1.42 ± 0.08*    |                    |                   |
| RBC count (x 106/mm/hr) |            |                   |
| 2.52 ± 0.02     | 2.34 ± 0.01*       | 2.26 ± 0.01*      |
| 2.17 ± 0.01*    |                    |                   |
| WBC count       |                    |                   |
| 58.35 ± 65      | 5445 ± 53*         | 5295 ± 47*        |
| 5250 ± 53*      |                    |                   |
The Statistical significance of difference between means were calculated by “Student’s test”. *p<0.05

**Table 2: Effect of protease from Ficus hispida on Differential leucocyte count in mice.**
(Values are mean-SEM of 10 experiments in each case)

| Parameters   | Water 5.0ml/kg b.w | Protease Solution |
|--------------|--------------------|-------------------|
|              | 136 mg/kg b.w      | 205mg/kg b.w      | 410 mg/kg b.w. |
| Neutrophil   | 18.0 ± 0.82        | 20.9 ± 0.94*      | 34.9 ± 0.82*   |
| Eosinophil   | 8.9 ± 0.28         | 9.5 ± 0.37        | 9.6 ± 0.43     |
| Basophil     | 1.6 ± 0.34         | 1.7 ± 0.26        | 1.4 ± 0.31     |
| Lymphocyte   | 55.1 ± 0.78        | 51.3 ± 1.04*      | 37.0 ± 0.92*   |
| Monocyte     | 16.4± 0.69         | 16.6 ± 0.70       | 17.1 ± 0.50    |

The statistical signigicance of difference between means were calculated by “Student’s test “. *p<0.05

**Table 3: Results of the wound healing response of protease from Ficus hispida on mice**

| Animal Group | Observation on day |
|--------------|--------------------|
| 1            | Necrosis, haemorrhage with RBC,LPC and PMNL |
| 2            | Necrosis, haemorrhage with RBC,LPC and PMNL |
| 3            | Necrosis, IFC (acute) PMNL |
| 4            | IFC (acute), PMNL |
| 5            | IFC (acute), PMNL |

| Observation on day |
|--------------------|
| 5                  |
| 10                 |
| 15                 |
| 20                 |

| LPC,PMNL,PC         | Formation of GT and SET |
| IFC,EPPTL, Beginning of KTN |
| Matured GT, EPTL   |
| Normal EPTL, KNT   |
| Normal EPTL, Normal KTN |

RBC – Red blood cells, LPC- Lymphocytes, PMNL – Polymorphonuclear leucocytes
IFC – Inflammatory cells, PC – Plasma cells, GT- Granulation tissue
SET – Subepithelial tissue, EPTL – Epithelialization, KTN – Keratinization

**Table 4: Anti-inflammatory activity of protease from ficus hispida on rats**

| Group No. of Treatment (One Observation in mm-SEM of 5 experiments after carragenan | Percent swelling | Percent inhibition of oedema formation |
|---------------------------------|-----------------|---------------------------------------|

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| rats | hour before carragenan injection | injection | 0hr ± | 1hr ± | 2hr ± | 3hr ± | 1hr ± | 2hr ± | 3hr ± | 1hr ± | 2hr ± | 3hr ± |
|------|---------------------------------|----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| I    | Control (Saline, 1.0ml/kg)      |          | 1.58  | 0.04  | 2.00  | 0.05  | 2.12  | 0.04  | 2.46  | 0.02  | 34.18 | 55.69 |
| II   | Diclofenac sodium (25.0mg/kg)   |          | 1.62  | 0.04  | 1.84  | 0.02  | 1.94  | 0.02  | 2.12  | 0.04  | 19.75 | 30.86 |
| III  | Protease                        |          | 1.64  | 0.02  | 1.94  | 0.02  | 2.06  | 0.02  | 2.36  | 0.02  | 25.61 | 43.90 |

Legend of the Figures:

Fig 1 Effect of protease from Ficus hispida on Haematological parameters in mice
(A) Clotting time (Sec)  (B) Haemoglobin content (%)
☐ - Water, ☐ - Low dose, ☐ - moderate dose, ☐ - High dose

Fig. 2 Effect of protease from Ficus hispida on Haematological parameters in mice
(C) ESR (mm/hr)  (D) RBC Count (X106/mm3)
☐ - Water, ☐ - Low dose, ☐ - moderate dose, ☐ - High dose

Fig. 3 Effect of protease from Ficus hispida on Haematological parameters in mice
(E) WBC Count (mm3)  (F) Differential leucocyte count (%)
☐ - Water, ☐ - Low dose, ☐ - moderate dose, ☐ - High dose

Fig 4 Histology of the wound tissue of mice treated for 10 days with ointment
Containing 0.5% (w/w) protease from Ficus hispida (Magnification x 400)

Fig 5 Histology of the wound tissue of mice treated for 10 days with ointment
Containing 1.0% (w/w) protease from Ficus hispida (Magnification x 400)

Fig 6 Histology of the wound tissue of mice treated for 20 days with ointment
Containing 1.0% (w/w) protease from Ficus hispida (Magnification x 400)
Fig. 1

(A) Clotting time (Sec)

(B) Haemoglobin content (%)

[Graphs showing data distribution]
Reduce about 60-75% to get more between plants

0.5% 10 days  
FIG - 4

1% 10 days  
FIG - 5

1% 20 days  
FIG - 6