CHAPTER IV

NEUTRALIZATION OF MALABARIN:
BETTER MANAGEMENT
OF
TRIMERESURUS MALABARICUS BITE
Introduction

From the systematic analysis it has been clearly established that protease are the major hydrolytic enzyme in *T. malabaricus* venom. Zymogram experiments revealed that *T. malabaricus* contain many isoforms (at least three) of protease enzymes. The major protease form purified from *T. malabaricus* venom is termed as malabarin. The substrate specificity of malabarin revealed that unlike other protease enzyme malabarin enzyme hydrolyzed wide variety of biologically important substrates such as fibrinogen and gelatin. These *in vitro* studies substantiated the observed pharmacological activities induced by *T. malabaricus* venom and indirectly suggested that the enzyme activity is essential for the induction of pharmacological activities.

Specific inhibition studies revealed that chelating agents inhibits the enzyme activities as well as neutralizes all the pharmacological activities induced by malabarin enzyme. Since all the pharmacological activities of crude *T. malabaricus* venom are also neutralized by specific protease inhibitors indirectly suggest that protease is the only toxic component of this venom. So effective inhibitions of this protease enzyme by inhibitors from medicinal plants or by antivenom are therapeutically important in the treatment of *T. malabaricus* snakebite.

In India there is no effective treatment available against *T. malabaricus* bite. The polyvalent antivenom raised cannot be used effectively because the polyvalent antivenom is not raised against *T. malabaricus* venom. The Indian Big Four snakes are not rich in protease enzyme and their involvement in the induction of toxic effects in those snakes are not prominent. Based on these observations one cannot expect any cross-reactions of the commercial antivenom against *T. malabaricus* venom. It requires a separate preparation of antiserum against *T. malabaricus* venom or one has to look for a specific protease inhibitor so that these inhibitors can be used as an alternate treatment.
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*p*-anisic acid has been shown to be a very good inhibitor of protease enzyme. In several cases *p*-anisic acid neutralizes effectively the hemorrhagic activity of *D. russelii* venom. In this chapter we have tried the inhibition of malabarin protease enzyme followed by the neutralization of pharmacological properties using *p*-anisic acid so that it can be used therapeutically against *T. malabaricus* snakebite. Alternatively antiserum is also raised against *T. malabaricus* venom in rabbits. This antiserum has been characterized for the effective neutralization of protease enzyme activity and its subsequent pharmacological activities.

Materials and Methods

Materials

Protease (Malabarin) enzyme purified from *T. malabaricus* venom as described in the chapter-III. Freund’s complete and incomplete adjuvants, agarose, *P*-anisic acid, vanillic acid, ajamaline, corynantheine, atropine, plumbagin and aristolochic acid were purchased from Sigma Chemical Company, St. Louis, MO, USA. 3,3’-diaminobenzidine tetrahydrochloride (DAB), goat anti-rabbit-IgG horseradish peroxidase, tetramethyl benzidine (TMB), nitrocellulose membrane, ponceau-S and Polyvinyl chloride microtitre plates were purchased from Genei Private Limited, India. Lactate dehydrogenase and creatine kinase kits were purchased from Auto Span Diagnostic Center, Bangalore, India. Inbreed male albino rabbit weighing around 2 kg and male Swiss Wistar albino mice weighing 20-25 g were obtained from Central Animal House facility, Department of Studies in Zoology, University of Mysore, Mysore, India. The animal care and handling were conducted in compliance with the national regulations for animal research. The animal experiments were carried out after reviewing the protocols by the animal ethical committee of the University of Mysore, Mysore.
Methods

Immunization of rabbit and preparation of *T. malabaricus* polyclonal antiserum

*T. malabaricus* venom (100 µg) in 0.2 ml PBS was mixed thoroughly with equal volume of Freund’s complete adjuvant and injected into male rabbit intradermally at several spots. Three booster doses with same concentration of *T. malabaricus* venom, mixed with equal volume of Freund’s incomplete adjuvant were administered at weekly intervals. A week after the last booster dose, blood was drawn from marginal ear vein and allowed to coagulate for 24 hr at 10 °C. The serum collected after centrifugation was set apart for further immunological studies.

Detection of antibodies

a) Ouchterlony immunodiffusion

The Ouchterlony immunodiffusion technique of Williams and Chase (1971) was used to detect antibodies. Diffusion plates were prepared by pouring 7 ml of 0.75 % molten agarose in 0.85 % NaCl solution into small petri dishes. After solidification, a central well and peripheral wells were made by cutting the agarose gel with the help of glass tube. The central well was filled with *T. malabaricus* antiserum (40 µl) and the peripheral wells were filled with various concentrations of malabaririn (25 µg and 50 µg). The plates were kept at 37 °C for about 48 hr. The precipitin line formed due to interaction of antigen and antibodies was photographed.

b) Enzyme linked immunosorbent assay (ELISA)

ELISA was performed according to the method of Middlebrook and Kaiser, (1989) with little modifications. Polyvinyl chloride microtitre plates were coated with malabaririn and *T. malabaricus* venom (100 ng / 0.1 ml) in 40 mM carbonate-bicarbonate buffer pH 9.6 and incubated over night at room temperature. The plates were washed five times thoroughly with PBST (PBS containing 0.05 % Tween 20). Last washing was done by leaving the buffer in the plate for 2 min. The buffer was completely removed by tapping the plate on tissue paper. The plates were blocked by
adding 0.1 ml of blocking solution (0.5 % of gelatin in PBST) and were incubated at 37 °C for 2 hr. Plates were washed five times in PBST. T. malabaricus antiserum of 0.1 ml serially diluted (in PBS) was added to each well and incubated at 37 °C for 2 hr. Unbound primary antibodies were washed five times in PBST. 0.1 ml of secondary antibody, anti rabbit IgG peroxidase conjugate diluted in PBS was added to each well and incubated for 2 hr at 37 °C. Unbound secondary antibodies were washed five times in PBST. Colour was developed by adding 0.1 ml of diluted substrate at room temperature (preferably in dark) for 30 min. The reaction was stopped by adding 50 μl of 1 N H₂SO₄ to each well. The colour complex was read at 450 nm using microplate reader (Medispec ELX 800).

**Preparation of T. malabaricus venom IgG**

Immunoglobulin G fraction was obtained from the antiserum raised against T. malabaricus venom by ammonium sulphate (NH₄₂SO₄) precipitation as described by Heide and Schwick (1973). To 20 ml of rabbit antiserum, NH₄₂SO₄ (5 g) was added pinch by pinch with constant stirring until the precipitation was formed at the final concentration of 2 M NH₄₂SO₄. The pH was then adjusted to 7.3 with 0.1 M NaOH and the mixture was left overnight and centrifuged at 10,000 g for 20 min and the supernatant was discarded. The precipitate was washed once with 2 M NH₄₂SO₄ solution. The pellet was evenly suspended in 1.75 M NH₄₂SO₄. This suspended solution was centrifuged at 10,000 g for 30 min. The pellet obtained was washed three times with 1.75 M NH₄₂SO₄ solution. The washed pellet was dissolved in small amount of water and dialyzed against distilled water using a dialysis bag with a molecular weight cutoff of 12,000 Da. The sample was further dialyzed against 80 mM sodium acetate buffer pH 5.0 and the precipitate formed during dialyses was removed by centrifugation at 10,000 g for 30 min. The clear supernatant was finally dialyzed against PBS and designated as T. malabaricus venom IgG. This fraction is used for neutralization studies.
Western blot analysis

*T. malabaricus* venom (75 μg) and malabarins (20 μg) samples were subjected to 10% SDS-PAGE under non-reduced conditions as described in chapter II. The separated proteins from gel were electro blotted onto nitrocellulose membrane. The membrane was blocked with 10% skimmed milk protein in TBS (Tris-buffered saline) for 2 hr. The membrane was washed thoroughly with TBST (TBS containing 0.2% Tween-20), and further incubated for 2 hr in TBST solution containing 1:100 diluted *T. malabaricus* antiserum. After washing, 5 times thoroughly in TBST, the membrane was incubated for 2 hr with peroxidase conjugated goat anti-rabbit IgG (1:5000 in TBS). The membrane was washed 5 times with TBST and the bands were visualized by the addition of substrate solution containing 3, 3′-diaminobenzidine tetrahydrochloride (DAB) according to the method of Harlow and Lane (1988).

Inhibition of protease activity

To determine the type of inhibition, Caseinolytic activity was assayed according to the method of Murata *et al.* (1963). 2 μg of malabarins and different concentrations of *p*-anisic acid and other compounds such as vanillic acid, ajamaline, corynantheine, atropine, plumbagin, ursolic acid and aristolochic acid were pre-incubated for 20 min. Reaction was initiated by adding 2% casein in 0.2 M Tris-HCl buffer, pH 8.5 and further incubated for 2 hr at 37 °C. One unit of enzyme activity was defined as the amount of enzyme required to increase an absorbance of 0.01 at 660 nm/hr at 37 °C. The inhibition data of *p*-anisic acid is expressed in percentage taking activity of malabarins alone as 100%.

Spectrophotofluorimetric interaction of malabarins with *p*-anisic acid

The fluorescence emission of malabarins with or without *p*-anisic acid was monitored using Shimadzu Spectrofluorophotometer. Malabarins (30 μg) was mixed with different concentrations of *p*-anisic acid in a total volume of 2 ml Tris-HCl buffer pH 8.0. Fluorescence emission was recorded between 300 to 400 nm by
exciting the proteins at 280 nm wavelengths. Measurements were made using 1 cm path length quartz cuvettes.

Inhibition of hemorrhage

Hemorrhagic activity was determined according to the method of Kondo et al. (1969) as described in Chapter II. The mice were divided into 5 groups. Samples were taken in a constant volume (20 μl) of saline injected by an intra dermal route on the back of mice. First group of mice received malabarin (3 μg) alone, the second group of mice received malabarin (3 μg) pre-incubated with \textit{T. malabaricus} IgG (90 μg; Ag: Ab, 1:30, w / w), the third group of mice received malabarin (3 μg) pre-incubated with \textit{p}-anisic acid (60 μg; enzyme: \textit{p}-anisic acid; 1:10, w / w) and fourth and fifth groups of mice received \textit{T. malabaricus} IgG (90 μg) and \textit{p}-anisic acid (60 μg) alone respectively. After 3 hr, mice were sacrificed using anesthesia (Borbitone 30 mg / kg i.p.). The dorsal surface of the skin was removed and diameters of hemorrhagic spots were measured and compared to those of control mice. Similarly, vanillic acid, ajamaline, corynantheine, atropine, plumbagin, ursolic acid and aristolochic acid compounds were tested for neutralization of hemorrhagic activity.

Inhibition of myotoxicity and histopathological studies

Myotoxicity in mice was measured by assaying the activity of serum CK and LDH enzymes according to the method of Hughes (1962) and King (1965) as provided in Auto Span Diagnostics kit. The mice were divided into 6 groups (n =3) and injected with the samples dissolved in 50 μl of saline and administered through intramuscular route. The first group of mice received saline, the second group of mice received malabarin alone (15 μg / 0.5 mg / kg body weight), the third group of mice received malabarin (15 μg) pre-incubated with \textit{T. malabaricus} IgG (150 μg; Ag: Ab, 1: 10, w / w), the fourth group of mice received malabarin (15 μg) pre-incubated with \textit{p}-anisic acid (360 μg; Enzyme: \textit{p}-anisic acid; 1:24, w / w), fifth and sixth groups of mice received \textit{T. malabaricus} IgG (150 μg) and \textit{p}-anisic acid (360 μg) alone respectively. After 3 hr, mice were anaesthetized and blood samples were collected.
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by cardiac puncture. The serum obtained was diluted 25 times with normal saline and assayed for CK and LDH enzyme activities. Activity was expressed as U/L.

For histopathological studies, muscle around the site of injection was removed, fixed in 10% formalin solution for 2 to 3 days and processed as described in chapter-II. The microphotographs were taken using an Olympus BX40F-3 microscope integrated with Olympus camera.

Inhibition of edema inducing activity

Edema inducing activity was determined by the method of Yamakawa et al. (1976) as modified by Vishwanath et al. (1987). The mice divided into 5 groups (n=3) were injected in the right footpads with the samples prepared in 20 μl saline. The left footpads received 20μl saline served as control. The first group of mice received 1 MED of malabarīn (1 μg), the second group of mice received malabarīn (1 μg) pre-incubated with T. malabaricus IgG (100 μg; Ag: Ab, 1:100, w / w), the third group of mice received malabarīn (1μg) pre-incubated with p-anisic acid (20 μg; Enzyme: p-anisic acid, 1:20, w / w) and the fourth and fifth group of mice received T. malabaricus IgG (100 μg) and p-anisic acid (20 μg) alone respectively. After 45 min, mice were sacrificed using anesthesia and the legs were removed at the ankle joints and weighed individually. The edema ratio was calculated using the formula.

\[
\text{Edema ratio} = \frac{\text{Weight of edematous leg} \times 100}{\text{Weight of normal leg}}
\]

Minimum edema dose is defined as the amount of protein required to cause an edema ratio of 120%.

Inhibition of Coagulant activity

Re-calcification time was determined according to the method of Condrea et al. (1983) as described in chapter II. Malabarīn (0.5 μg) was pre-incubated for 20 min with different concentration of T. malabaricus IgG (1:2, 1:4, 1:6 and 1:8; w / w) and p-anisic acid (1:3, 1:6, 1:9 and 1:12; w / w) in 10 mM Tris-HCl buffer pH 7.4.
These pre-incubated mixtures were separately added to 0.3 ml of PPP and incubated for one min. The clot formation was initiated by adding 30 µl of 0.25 M CaCl₂ and the time taken for visible clot to appear from the time of addition of CaCl₂ was recorded in sec. For control experiments Tris-HCl buffer with T. malabaricus IgG (1:8) or p-anisic acid (1:12) was added instead of the venom sample.

**Statistics**

The data were expressed as the mean ± SEM of at least three independent experiments.

**Result and discussion**

Snakebite is a global problem, especially in tropical countries like India. In Asia, as a whole, there may be up to four million snakebites every year, of which almost 50 % are envenomed. It was estimated in 1996 that annual snakebite mortality in Indian subcontinent is more than 25,000 (Warell, 1996). Even now the mortality rate has been found to be the same. Snake envenomation involve subcutaneous or intramuscular injection of venom into the prey / human victims. The pathology of envenomation includes both local and systemic effects (Warell, 1996; Schneemann et al., 2004). Local effects which includes severe inflammatory reactions; hemorrhage and local tissue necrosis are among the most dramatic effects of envenomation by crotalinae and viperinae snakes (Gutierrez and Rucavado, 2000).

Envenomation by snakebites are frequently treated by the parenteral administration of horse or sheep - derived antivenoms aimed at the neutralization of toxins (Warrell. 1996; Leon et al., 1998). Antiserum, the only remedy for envenomation, apart from its high cost and unavailability, at times does not provide enough protection against venom induced hemorrhage, hemostatic alterations, neurotoxicity and local tissue damage (White, 2005; Stephano et al., 2005). In addition, antiserum therapy is associated with various reactions viz, early anaphylacoid reaction, pyrogenic reaction and late serum sickness reactions, besides several other manifestations (Singh et al., 2001; Offerman et al., 2001; Holstege et
Considering all these drawbacks, antiserum therapy is the preferred therapy in the hospitals to treat snakebite. Malabarin protease from *T. malabaricus* has been characterized as the main toxin responsible for the induction of extensive local tissue damage consisting of edema, hemorrhage and myotoxicity. Malabarin also exhibit strong procoagulant activity and has been speculated to be the reason for the observed myotoxicity. Neutralization of malabarin protease by raising antiserum or by specific inhibitor is therapeutically very important.

*T. malabaricus* venom has been used to raise polyclonal antibody in rabbits. Ouchterlony immunodiffusion, ELISA, and Western blot methods are being extensively used to examine the antigenic relationship among snake venom components (Basavarajappa et al., 1993; Howes et al., 2005; Girish and Kemparaju, 2005). The *T. malabaricus* polyclonal antiserum prepared in rabbit gave a single antigen: antibody precipitin band with malabarin in Ouchterlony immunodiffusion (Figure 4.01). On the other hand, the pre-immune serum did not show any precipitin line (data not shown). Figure 4.02 show the immunoreactivity of malabarin and *T. malabaricus* venom by ELISA method. The *T. malabaricus* showed high immunoreactivity with a titer value of 1/18,000, while malabarin showed a titer value of 1/12,000. In contrast, the pre-immune serum did not show any immune reactivity with no titer value when added directly. The titer values indirectly suggests that malabarin in *T. malabaricus* is highly antigenic in nature. Further, immunoreactivity of *T. malabaricus* polyclonal antiserum was substantiated by Western blot. *T. malabaricus* venom showed 4-5 distinct bands where as malabarin showed single clear band demonstrating the purity of the malabarin protease enzyme (Figure 4.03).

Studies with *T. malabaricus* whole venom and purified malabarin protease revealed that the toxicity of *T. malabaricus* venom is due to the protease action of malabarin. The success of any polyclonal antibody raised against snake venom lies in the effective neutralization of pharmacological activities induced by respective venom toxins. The inhibition of enzyme activity and subsequent neutralization of
pharmacological activities of *T. malabaricus* whole venom and purified malabar in protease by antiserum is further studied.

The pharmacological or toxicological properties of snake venom are mainly associated with proteins, particularly with enzymes (Stocker, 1990; Markland Jr, 1997; Markland, 1998; Soares *et al.*, 2005; Lu *et al.*, 2005). Some of the local pathological effects of envenomations, such as myonecrosis and muscular degeneration, are due to the presence of proteases in snake venom (Rucavado *et al.*, 2002; Laing *et al.*, 2003; Neto and Marques, 2005). Malabar in exhibited significant protease activity at 2 μg protein concentration. *T. malabaricus* IgG inhibited the proteolytic activity of both malabar in and *T. malabaricus* venom in a dose dependent manner and complete inhibition of proteolytic activity was seen at an Ag: Ab ratio of 1:12 (w / w) for malabar in and 1:7 (w / w) ratio of *T. malabaricus* venom respectively (Figure 4.04). The immune complex formed between venom and antiserum resulted in the complete inhibition of protease activity.

However, despite the widespread success of antivenom therapy, it is important to search for different venom enzyme inhibitors, either synthetic or natural, that would complement the action of antivenoms, particularly regarding neutralization of local tissue damage. Plant extracts constitute an extremely rich source of pharmacologically active compounds and a number of them have been shown to posse's antivenom activities (Alam and Gomes, 1998; Uma and Gowda, 2000; Alam and Gomes, 2003; Girish *et al.*, 2004).

Over the years, many attempts have been made for the development of chemical antagonist, which may provide protection against snakebite. Not many compounds are known even today which are proved to be satisfactory to counter venom action, except for a few observations (Pereira *et al.*, 1994; Mors *et al.*, 2000). Gomes *et al.* (1994) have reported the neutralization of lethal, hemorrhagic, coagulant and anticoagulant activities of *V. russelii* venom by a plant isolate HI-RVIF (2-hydroxy-4-methoxy benzoic acid) from the root extract of an Indian medicinal plant.
Chapter 4 Neutralization of Malabarinsarsaparilla (*Hemidesmus indicus*). Anisic acid (4-methoxy benzoic acid) a structural analog of HI-RVIF was also effective against *V. russelii* venom induced pharmacological effects (Alam and Gomes, 1998). *p*-anisic acid an effective inhibitor of venom induced pharmacological activities has been checked for the inhibition of protease activity of *T. malabaricus* venom and purified malabarin protease. When 5 µg of malabarin was pre-incubated with different ratios of *p*-anisic acid (10 -50 µg; 1:0- 1:10; w / w) a dose dependent inhibition of proteolytic activity was observed. At 1:2.7 (w / w) ratio of *p*-anisic acid a 50 % (IC₅₀) inhibition was observed. Whereas 80 - 90 % of the activity was abolished at 1:8 (w / w) ratio of *p*-anisic acid as shown in figure 4. 05.

To study the mechanism of inhibition of malabarin protease activity by *p*-anisic acid fluorescent interaction studies was employed. The change in the intrinsic fluorescence reflects conformational changes in proteins due to ligand binding. Thus it was expected that fluorescence measurements of malabarin with *p*-anisic acid would provide information of inhibitor binding directly with the enzyme. Malabarin showed tyrosine specific fluorescence emission maxima between 300 - 320 nm and tryptophan specific emission maximum between 330 - 350 nm. The added *p*-anisic acid quenched the pronounced fluorescence intensity of malabarin and the quenching was found to increase with increasing concentration (Figure 4. 06). The quenching of malabarin with *p*-anisic acid indicates the formation of enzyme-inhibitor complex. Since antiserum raised against whole *T. malabaricus* venom and *p*-anisic acid inhibits the protease activity of *T. malabaricus* venom and purified malabarin protease effectively, its subsequent neutralization of pharmacological activities was studied.

Coagulopathy is one the major complications following Viperidae snakes envenomation. The venom of such snakes contains thrombin-like enzymes and procoagulants (Boyer *et al.*, 1999; White, 2005). Procoagulants activate coagulation cascade in the victims while thrombin-like enzymes induce direct clotting of fibrinogen. These components lead to consumption of clotting factors (Braud *et al.*, 2000; Lu *et al.*, 2005; White, 2005). However, snake envenomation is considered as a
subcutaneous or intramuscular injection of the venom. To allow the development of coagulopathy, these venom coagulation enzymes are needed to spread from the injected area into systemic circulation. Based on the enzymatic properties of venom hemorrhagic metalloproteinases, it is stated that it may be involved in the rapid spreading of venom components from injected area into systemic circulation (Chen et al., 2005). Malabarin at 0.5 µg protein concentration induced strong procoagulant effect on platelet poor plasma and clot formation was observed with in 10 ± 0.9s when compared to the normal clotting time. The T. malabaricus IgG inhibited clotting time in a dose dependent manner and at a ratio of 1:8 (w / w), the clotting time of malabarin was almost equivalent to normal clotting time of plasma as shown in Figure 4. 07A. Similarly, p-anisic acid also inhibited the procoagulant activity of malabarin in a dose dependent manner and at a ratio of 1:12 (w / w), the clotting time was almost normal as shown in Figure 4. 07B.

Apart from coagulation enzymes, the venom also contains hemorrhagic metalloproteinases, known as hemorrhagic factors or hemorrhagins. They cause local tissue damage characterized by hemorrhage, edema and necrosis (Laing et al., 2003; Chen et al., 2005; Farsky et al., 2005). The underlying mechanism is the enzymatic degradation of basement membrane and extracellular matrix surrounding capillaries and small vessels (Hati et al., 1999). Hemorrhagic is one of the most prevalent signs of local and systemic envenomation induced by T. malabaricus (Gowda et al., 2006). The malabarin exhibited MHD at 3 µg concentration. When malabarin was co-injected after pre-incubated with T. malabaricus IgG (1:12, w / w) or p-anisic acid (1:13, w / w), a complete neutralization of hemorrhagic activity was observed. The T. malabaricus IgG (90 µg) and p-anisic acid (60 µg) did not induce any hemorrhagic spot as shown in Figure 4. 08.

Swelling and edema are the early clinical features observed in snake venom poisoning at the affected part of the victim. The edema induction is due to the increased vascular permeability, which is mediated by the release of autocoids such as histamine, serotonine, kinins, prostoglandins and leukotriens (Teixeira et al., 2005;
Chen et al., 2005; Farsky et al., 2005). Edema inducing activity is a multifactorial pharmacological activity, depending on the combined action of various toxins, suggesting that enzymatic activity is not strictly required to induce this effect (Barbosa et al., 2003). Malabarin induced hemorrhagic edema of 180 % at 1 μg protein concentration. T. malabaricus IgG (1:7; w / w) and p-anisic acid (1:5; w / w) was capable in inhibiting the edema inducing activity of malabarin and the edema ratio of 180 ± 0.41 was reduced to 118 ± 0.76 as shown in the Figure 4. 09.

Envenomations by snakes of the family viperidae usually causes local tissue damage, including edema, hemorrhage and myonecrosis, drastic effects that are only partially neutralized by conventional serotherapy. In severe cases, such effects may lead to permanent tissue loss, disability or amputation (Chattopadhya et al., 2004). Myonecrosis is caused by irreversible cell damage to skeletal muscle fibers due to the action of venom components that directly affect the integrity of their plasma membrane. In addition, myonecrosis can be secondary to the ischemia that results from the action of venom hemorrhagic metalloproteinases (Gutierrez and Rucavado, 2000; Gutierrez et al., 2005). On the other hand, hemorrhage develops due to the proteolytic action of zinc-dependent metalloproteinases upon basal lamina components of the microvasculature leading to endothelial cell distension and rupture (Gutierrez et al., 2005). The striking observation with malabarin (15 μg; 0.5 mg / kg body weight) when intramuscularly injected to experimental animals is the extensive degradation of muscle tissue with in 3 hr as shown in histopathological studies. T. malabaricus IgG (150 μg; 1:10; w / w) upon co-injection with malabarin completely neutralized the necrotic symptoms with in 3 hr. Where as, p-anisic acid did not significantly neutralizes the tissue necrosis even at (360 μg; 1:24; w / w) concentration. The T. malabaricus IgG (150 μg) and p-anisic acid (360 μg) alone did not induce local tissue damage as shown in Figure 4. 10. The extent of muscle degeneration is further supported by the marked increase in CK and LDH levels. The malabarin exhibited significant increase in CK values up to 22,000 U / L. Similarly, the LDH values were also increased to 12,000 U / L after 3 hr of injection. When
malabarins was pre-incubated with *T. malabaricus* IgG (150 μg; 1:10; w / w) and p-anisic acid (360 μg; 1:24; w / w) for 20 min and co-injected to group of mice, *T. malabaricus* IgG was more potent in reducing CK and LDH values when compared to p-anisic acid. The *T. malabaricus* IgG (150 μg) and p-anisic acid (360 μg) did not induce myotoxic activity as shown in Figure 4.11.

These neutralization studies suggest that antiserum raised in rabbit forms antigen-antibody complex. This complex resulted in the inhibition of protease enzyme activity of crude venom and purified malabarins protease enzyme and subsequently neutralized all the pharmacological activities induced by them. These studies supported that the local tissue damages are due to protease enzyme activity. In *T. malabaricus* venom the principle toxin is malabarins, a protease enzyme responsible for the induction of edema, hemorrhage, procoagulant action and myotoxicity. Specific inhibitors of venom enzymes are clinically relevant and would complement the action of antiserum therapy.
Figure 4.01: Ouchterlony immunodiffusion of malabar in with *T. malabaricus* polyclonal antiserum

Immunodiffusion was carried out using 0.75% agarose gel. Central well contained 100 μl of *T. malabaricus* polyclonal antiserum and the peripheral wells A and B were loaded with 25 and 50 μg of malabar in respectively.
Figure 4.02: Immunoreactivity of malabar in and *T. malabaricus* venom with *T. malabaricus* polyclonal antiserum in ELISA

Each well in microtiter plate was coated with 100 ng of malabar in or 200 ng of *T. malabaricus* venom, before incubating with *T. malabaricus* polyclonal antiserum. Further 5000 times diluted goat anti-rabbit IgG conjugated with peroxidase and substrate TMB / H₂O₂ were added and read at 450 nm. Values are mean ± S.E.M of three experiments.
Figure 4.03: Immunoblot of *T. malabaricus* venom and malabarin

60 µg *T. malabaricus* venom (lane 1) and 20 µg of malabarin (lane 2) were electrophoresed on 10% SDS-PAGE under non-reducing condition and transferred to nitrocellulose membrane. Membrane incubated with 100 times diluted *T. malabaricus* polyclonal antiserum Bands were visualized after incubating with anti rabbit IgG conjugated with peroxidase enzyme. M = Markers in kDa, (Phosphorylase b (97.4), bovine serum albumin incubation with (66.0), ovalbumin (43.0), carbonic anhydrase (29.0), soyabean trypsin inhibitor (20.0) and lysozyme (14.3).
10 μg T. malabaricus venom and 2 μg malabar in were pre-incubated separately with different concentrations of T. malabaricus IgG (w / w) for 20 min at 37 °C. The mixture was incubated with casein substrate and incubated for 2 hr at 37 °C. The protease activity in the absence of inhibitor was considered as 100 %. Values represent mean ± S.E.M of three experiments.

Figure 4. 04: Inhibition of Protease activity of T. malabaricus venom and malabar in by T. malabaricus IgG
Figure 4.05: Inhibition of protease activity of malabarín by $\text{p}$-anisic acid

2 μg of malabarín was pre-incubated with 0 - 50 μg of $\text{p}$-anisic acid for 20 min at 37 °C. The mixture was incubated with casein substrate for 2 hr at 37 °C. The activity in absence of $\text{p}$-anisic acid was considered as 100 %. Values represent mean ± S.E.M of three experiments.
Figure 4.06: Fluorescence emission spectrum of malabar in presence of \textit{p}-anisic acid

Fluorescence emission of 30 \( \mu \text{g} \) malabar in was measured in the absence (A) and presence of different concentrations of \textit{p}-anisic acid in 2 ml of Tris-HCl buffer pH 8.0. The solutions were excited at 280 nm and emission was recorded between 300-400 nm. Values represent mean ± S.E.M of three experiments. B-10 \( \mu \text{g} \) \textit{p}-anisic acid, C-20 \( \mu \text{g} \) \textit{p}-anisic acid, D- 30 \( \mu \text{g} \) \textit{p}-anisic acid, E- 40 \( \mu \text{g} \) \textit{p}-anisic acid, F-10 \( \mu \text{g} \) \textit{p}-anisic acid and G- 50 \( \mu \text{g} \) of \textit{p}-anisic acid.
Figure 4.07: Effect of *T. malabaricus* IgG and p-anisic acid on coagulant activity of malabar

0.5 µg malabar was pre-incubated with different concentrations of *T. malabaricus* IgG (A) and p-anisic acid (B) for 20 min at 37 °C. The pre-incubated mixture was added to 0.3 ml platelet poor plasma at 37 °C and incubated for 1 min. 30 µl CaCl$_2$ (0.25 M) was added to the mixture to initiate the clot formation. Values represent Mean ± S.E.M. of three experiments.
Figure 4.08: Inhibition of Hemorrhagic activity of malabar in by *T. malabaricus* IgG and *P*-anisic acid

3 μg of malabar in preincubated with or without the inhibitors in a total volume of 20 μl. The mixtures were injected intradermally to mice the skin was removed after 3 hr and the extent of hemorrhage was photographed. [A] Saline; [B] 3 μg malabar; [C] malabar: *T. malabaricus* IgG (1:30, w/w); [D] malabar: *p*-anisic acid (1:10); [E] 90 μg *T. malabaricus* IgG alone and [F] 60 μg *p*-anisic acid alone.
Figure 4. 09: Effect of *T. malabaricus* IgG and *p*-anisic acid on edema inducing activity of malabarin:

1 μg of malabarin in 15 μl of saline was injected with or without pretreated inhibitors into intra plantar surface of hind limb of mice. After 45 min legs were removed at ankle joint and edema was calculated. [1] 1 μg malabarin; [2] malabarin: *T. malabaricus* IgG (1:15, w/w); [3] malabarin: *p*-anisic acid (1:10); [4] 15 μg *T. malabaricus* IgG alone and [5] 10 μg *p*-anisic acid alone.
Figure 4.10: Histopathological section of malabarin incubated with T. malabaricus IgG and p-anisic acid

Muscle around the site of injection was removed, fixed in 10% formalin solution for 2 to 3 days and further processed as described in chapter-II. [A] Saline; [B] malabarin alone (0.5 mg / kg body weight); [C] malabarin: T. malabaricus IgG (1: 10, w/w); [D] malabarin: p-anisic acid (1:24); [E] T. malabaricus IgG (150 µg) alone and [F] p-anisic acid (360 µg) alone.
Figure 4.11: Effect of *T. malabaricus* IgG and *p*-anisic acid on the serum CK and LDH activity in mice after intramuscular injection of malabarin:

Samples were injected at a constant volume of 50 μl in saline by an intramuscular route on the right thigh of mice and myotoxicity level was carried out as described in methodology section. Serum CK and LDH activity were assayed using Span diagnostics kit. The activity was expressed in U/L. Values are mean ± S.D. of three independent experiments. The Animals were injected with [1] saline; [2] malabarin alone (15 μg / 0.5 mg / kg body weight); [3] malabarin (15 μg), which was pre-incubate with *T. malabaricus* IgG (150 μg; Ag: Ab, 1:10, w/w); [4] malabarin (15 μg), which was pre-incubated with *p*-anisic acid (360 μg; Enzyme: *p*-anisic acid, 1:24, w/w); [5] *T. malabaricus* IgG (150 μg) alone and [6] *p*-anisic acid (360 μg) alone.
Table 4.01: Effects of compounds on protease activity of malabarlin

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (μM) (%) of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 μM</td>
</tr>
<tr>
<td>Ajmaline</td>
<td>6.87 ± 0.3</td>
</tr>
<tr>
<td>Aristolochic acid</td>
<td>0</td>
</tr>
<tr>
<td>Atropine</td>
<td>3.34 ±1.3</td>
</tr>
<tr>
<td>Corynantheine</td>
<td>5.78 ±0.9</td>
</tr>
<tr>
<td>α-anisic acid</td>
<td>87.34 ± 3.1</td>
</tr>
<tr>
<td>Plumbagin</td>
<td>7.98 ± 1.9</td>
</tr>
<tr>
<td>Ursolic acid</td>
<td>9.45 ± 0.5</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>3.24 ± 1.6</td>
</tr>
</tbody>
</table>

Malabarlin (2 μg) was preincubate separately with inhibitors (50 μM and 100 μM) for 20 min at 37 °C. Values are mean ± SEM of five experiments.
Table 4.02: Effect of compounds on hemorrhagic activity of malabar in

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Hemorrhagic spot (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0</td>
</tr>
<tr>
<td>Malabar in (3 μg) + Ajmaline (100 μg)</td>
<td>13.0 ± 0.15</td>
</tr>
<tr>
<td>Malabar in (3 μg) + Aristolochic acid (100 μg)</td>
<td>12.9 ± 0.23</td>
</tr>
<tr>
<td>Malabar in (3 μg) + Atropine (100 μg)</td>
<td>13.1 ± 0.02</td>
</tr>
<tr>
<td>Malabar in (3 μg) + Corynantheine (100 μg)</td>
<td>12.4 ± 0.12</td>
</tr>
<tr>
<td>Malabar in (3 μg) + p-anisic acid (100 μg)</td>
<td>0</td>
</tr>
<tr>
<td>Malabar in (3 μg) + Plumbagin (100 μg)</td>
<td>13.1 ± 0.09</td>
</tr>
<tr>
<td>Malabar in (3 μg) + Ursolic acid (100 μg)</td>
<td>13.0 ± 0.12</td>
</tr>
<tr>
<td>Malabar in (3 μg) + Vanillic acid (100 μg)</td>
<td>12.6 ± 0.31</td>
</tr>
</tbody>
</table>

Values are mean hemorrhagic diameters (mm) ± SEM.