Effects of Tamarind (*Tamarindus indicus* Linn) seed extract on Russell's viper (*Daboia russelli siamensis*) venom

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Abstract. Snake bite has been regarded as an important health problem in Myanmar since early 1960's. In the recent years, there has been growing interest in alternative therapies and therapeutic use of natural products, especially those derive from plants. In Myanmar and Indian traditional medicine, various plants have used as a remedy for treating snake bite. The present study was carried out to evaluate the effects of alcohol extract of Tamarind (*Tamarindus indica* Linn.) seed on some biologic properties of Russell's viper (*Daboia russelli siamensis*) venom (RVV). The Phospholipase A_2 (PLA_2) enzyme, coagulase enzyme and caseinolytic enzyme activities of Russell's viper venom (RVV) were reduced when mixed and incubated with the extract. When the RVV and the different amount of extracts were preincubated and injected intramuscularly into mice, all of them survived, but all the mice in the control group died. On the other hand, when RVV were injected first followed by the extract into mice, all of them died. If the extract was injected near the site where Russell's viper venom was injected, all the mice survived for more than 24 hours and the survival time prolonged but they all died within 96 hours. In conclusion, according to the results obtained, the extract neutralizes some biologic properties of the Russell's viper venom and prolonged the survival time if the extract was injected near the site where the Russell's viper venom was injected.

INTRODUCTION

Antivenom is currently the only available antidote for treating snake bite universally. However, untoward effects are usually associated with its use and there are poor neutralization of the local or even systemic effects of venom (WHO, 1981; Warrell, 1993). Therefore, an alternative treatment for snake bites is needed as an add-on to antivenom treatment or as a stand-alone treatment. Studies to develop more effective therapeutic strategies for the treatment of snake envenomation have been on-going for many decades. Over the years many attempts have been made for the development of snake

venom antagonists especially from plant sources (Sanchez *et al.*, 2008).

In India, the rural areas are most affected by snake envenomation and medicinal plants have been widely used as a remedy for treating snake bite (Panghal *et al.*, 2010). In the recent years, there has been growing interest in alternative therapies and therapeutic use of natural products, especially those derived from plants and various plants have been reported to have detoxifying effects on snake venom (Otero *et al.*, 2000). Several studies have shown the neutralization of the main effects induced by snake venoms using plant extracts (Melo *et*

al., 1994; Borges et al., 2000, 2001, Asuzu & Harvey, 2003).

Tamarind (*Tamarindus indica*) is widely found in many Asian countries including India and Myanmar and its uses in the treatment of snake bite can be found in Myanmar traditional medicine books. However, scientific studies have not been done in Myanmar. Therefore the present study was to evaluate the neutralization capacity of tamarind seed extracts against some biochemical properties of lyophilized Russell's viper venom (RVV).

MATERIALS AND METHODS

Tamarind (from central Myanmar) used for cooking purpose was bought from the market and the seeds collected for use in this study. Lyophilized Russell's viper venom (RVV) was kindly supplied by the Myanmar Pharmaceutical Factory (MPF). Chemicals of the Analar grade from the Sigma Chemical Company were used. Male and female mice, Institute of Cancer Research (ICR) strain of 20 ± 2 g weight were obtained from the Laboratory Animal Service Division, Department of Medical Research Lower Myanmar.

Preparation of tamarind seed coat extract (TSE)

The tamarind seed coats were separated from the endosperm and were finely powdered and 10 g of the powder was subjected to Soxhlet extraction using 125 ml of 99.7% ethanol. The solvent was removed by warming at 65°C in the water-bath. The dried extract of tamarind seed (TSE), was dissolved in distilled water (10 mg /ml) and was used for the studies on the activities of the RVV enzymes and its neutralization properties.

Determination of Phospholipase A_2 (PLA_2) enzyme activity of RVV solution

The determination of the activity was carried out by a slight modification of the methods by Araujo & Radvanyi (1987). A substrate solution (3.5mM Phosphotidyl choline in a solution containing 7mM Triton X100, 100mM NaCl, 10mM $CaCl_2$ and pH adjusted to 6.8 with N/100 NaOH) was mixed with 10 µl of RVV sample- (250 µg /ml) and incubated at 37°C for 10 minutes. The reaction was stopped with one ml of acetone-ethanol solution (1:1v/v). The mixture was centrifuged at 2000 rpm for 5 minutes, supernatant collected. The action of the Phospholipaes A_2 enzyme produces fatty acid and the amount of fatty acid in the reaction mixture was determined by titration with N/100 NaOH solution to pH 6.8. The amount of NaOH used was noted and taken as PLA_2 enzyme activity (greater the enzyme activity more acid will be produced). To determine the inhibitory action of TSE, 1ml of substrate solution and different volumes (100, 500, 1000 and 1500 µl) of TSE solution (10mg/ml) with pH 6.8 were combined and used as the substrate inhibitor mixture for the determination of PLA_2 activity of 10 µl of RVV solution (250 µg/ml) as described above.

The amount of N/100 NaOH used for substrate solution without inhibitor was taken as 100% enzyme activity and percentage of enzyme activity with TSE solution relative to control (RVV only) was calculated as follows:

Percentage activity relative to control = (Vol. of NaOH used in the presence of RVV & TSE solution) / (Vol. of NaOH used in the presence of RVV only) x 100.

The results were shown as mean of triplicate determinations.

Determination of coagulase enzymes activity of RVV solution

The method of Dimitrov & Kankonkar (1968) was used for the determination of coagulase enzyme activities. One hundre µl of human plasma was collected with 3% sodium citrate solution and mixed with 100 µl of Tris saline buffer (pH7.3)(0.01M Tris in 0.15M NaCl) and 40µl of 0.05 M $CaCl_2$. The reaction mixture was observed for the formation of clot. The time taken for formation of clot (in seconds) was taken as enzyme activity. To know the activities of RVV, 40 µl of RVV solution was added to human plasma and Tris buffer mixture before $CaCl_2$ solution was added and

time taken for the formation of clot was noted. To know the inhibition effect of TSE solution, different dilutions of it (i.e., 10, 20, 30, 40, 50 µl) were added to reaction mixture described above and incubated at 37ºC for 10 minutes. After this, $CaCl_2$ solution was added and the time taken for formation of clot was noted. The coagulase enzyme activity of RVV solution was taken as control (100%) and the percentage of enzyme activity of the reaction containing different volume of TSE solution, relative to control was calculated as follows:

Percentage activity relative to control = (Reaction time (sec) of RVV solution only)/ (Reaction time of RVV-TSE mixture) x 100.

The results were shown as mean ± SD of triplicate determinations.

Determination of caseinolytic enzyme activity of RVVsolution

The caseinolytic enzyme activity was determined by the method of Kunitz (1947). One ml of substrate solution (1% casein in 0.1M phosphate buffer pH7.0) and 50 µl of RVV solution (1mg/ml) were mixed and incubated at 37ºC for 15 minutes. The reaction was stopped by adding 2 ml of 5% Trichloroacetic acid solution and allowed to stand for 30 minutes at room temperature. The mixture was centrifuged at 10000 rpm for 5 minutes and the precipitate collected and washed with 5% TCA solution. Then the precipitate was dissolved in 1N NaOH solution and absorbance measured at 280nm wavelength. For determination of inhibitory activity of TSE solution, different volumes of it (i.e., 125, 250, 375, 500 µl) were mixed with the substrate solution and RVV solution and continued as above. Absorbance of the substrate solution was determined by using distilled water 50 µl instead of RVV solution and continued as described above. The difference between absorbance of substrate solution only and absorbance of the reaction mixture (substrate and RVV solution) was taken as activity of RVV solution. The difference of absorbance of substrate solution only and absorbance of reaction mixture and TSE solution were taken as inhibitory activity of TSE. Percentage of caseinolytic activity of RVV: TSE mixture is calculated as follows.

Percentage activity relative to control = (Absorbance of substrate solution – absorbance of reaction mixture) / (Absorbance of substrate solution – absorbance of reaction mixture with TSE solution) x 100.

Results were shown as mean ± SD of triplicate determinations.

Determination of neutralization potency of Tamarind seed extract (TSE) against lethal action of Russell's viper venom RVV (injection of mixture of RVV and TSE)

One hundred microlitre of RVV solution (containing 166 µg of RVV, the amount which is equivalent to 2 LD_{50} dose of RVV) was injected intramuscularly (IM) into the inner side of the thigh of the control group mice. One hundred microlitre of RVV solution and different amount of TSE (1:1, 1:5, 1:20 times the weight of RVV) were mixed and incubated for 30 minutes at 37ºC and injected IM into the inner side of the thigh of the test groups mice. Five mice were used in each group and they were observed for 24 hours.

Determination of neutralization potency of Tamarind seed extract (TSE) against lethal action of RVV (injecting venom and TSE separately)

One hundred microlitre of RVV solution (containing 166 µg of RVV, the amount which is equivalent to 2 LD_{50} dose of RVV) was injected IM into the inner side of the right thigh of both control group and test group mice. After 15 minutes, for the test group, 100 µl TSE solution containing 20 times the weight of RVV was injected IM into the left thigh and for the control group 100 µl of distilled water was used. Five mice were used in each group and they were observed for 24 hours.

Determination of affect of local infiltration of the tamarind seed extract on the lethal action of RVV

One hundred microlitre of RVV solution (containing 166 µg of RVV, the amount which

is equivalent to 2 LD_{50} dose of RVV) was injected IM into the inner side of the thigh of both control and test group mice. After 30 minutes, for the test group, 100 µl TSE solution containing 20 times the weight of RVV was injected IM into the thigh near the site where RVV was injected previously. For the control group 100 µl distilled water was used. Each group had 3 mice and they were observed for 96 hours.

RESULTS AND DISCUSSION

Snake venom is a complex mixture of different classes of hydrolytic enzymes such as Phospholipase A_2, metalloproteinase, hyaluronidase, L amino oxidase etc. PLA_2 enzyme are the most abundant class of proteins in snake venom especially Russell's viper venom. Aye Kyaw *et al.* in 1994 had purified and studied PLA_2 of RVV and showed that it had properties that could cause oedema; myonecrosis and indirect hemolysis. PLA_2 of RVV constitutes of 12% total protein and its effect is 12 fold greater than that of the whole venom (Maung *et al.*, 1995). The TSE on the PLA_2 enzyme activity of RVV was studied and found to inhibit the enzyme activity in a dose related fashion (Figure 1).

Just as in PLA_2, RVV contain two coagulase enzymes, namely Factor V activator and Factor X activator, which act on the blood coagulation system of snake bite victims giving rise to various pathological conditions leading to death of patients (Than *et al.*, 1987). To know the effect of TSE on coagulase enzymes of RVV, experiments were conducted with human plasma, RVV and TSE. The reaction time (time taken to for blood clot) of the plasma only (without RVV or TSE) was 365 seconds and that of the plasma and TSE (20 ml) was 365 seconds. This shows that TSE had no action on coagulase enzymes of human plasma and that it only inhibited the coagulase enzymes activity of RVV. From Figure 2, it can be seen that TSE inhibits the
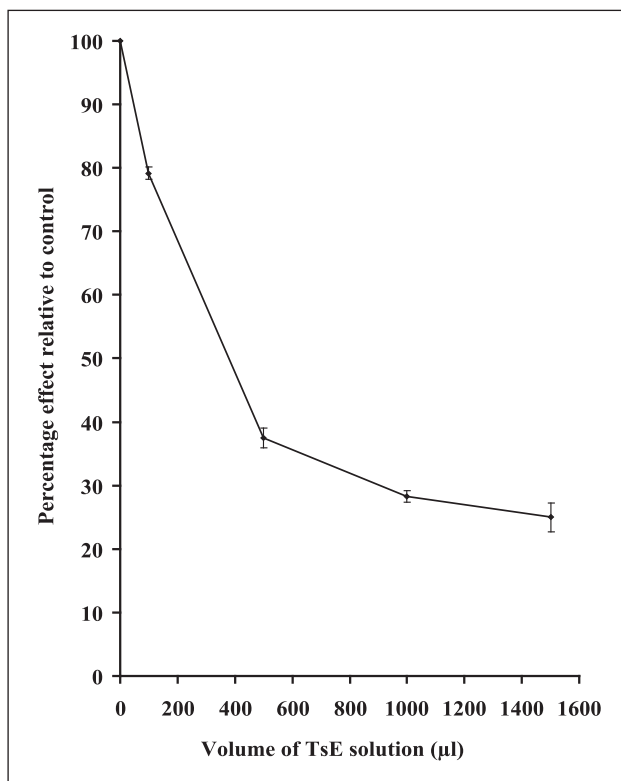

Figure 1. Inhibitory effect of tamarind seed extract on PLA_2 enzyme of desiccated Russell's viper venom
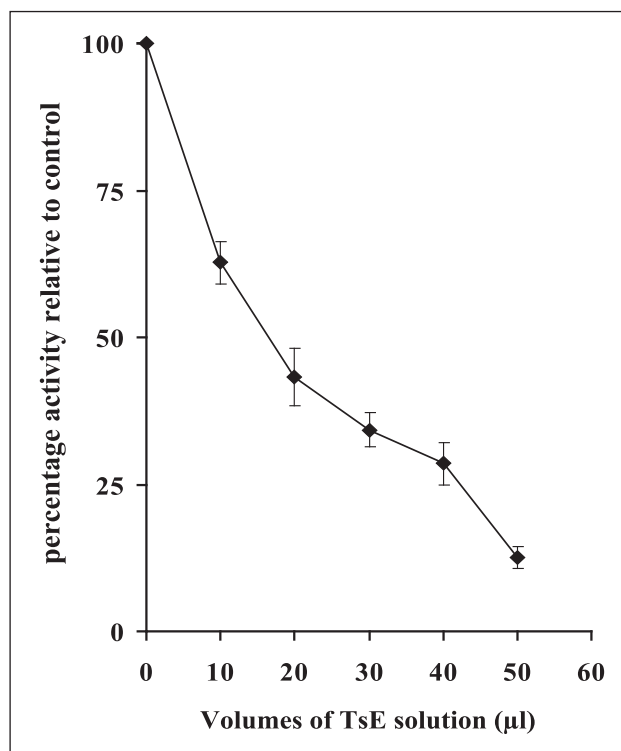
Figure 2. Effect of tamarind seed extract on coagulase enzyme of Russell's viper venom

coagulase enzymes of RVV in dose dependent fashion. As blood coagulating factors are serine proteases, TSE may inhibit the activity of other serine proteases.

To find the effect on other proteases, caseinolytic enzyme (proteinase) activity was tested with TSE. From Figure 3, it can be seen that caseinolytic enzyme activity was inhibited by TSE. Tu *et al.* (1967) showed that there is a correlation between proteolytic enzyme activity and hemorrhagic activity of the venom. Proteolytic enzyme will hydrolyse different proteins in human victims, leading to the various pathological processes due to snake bite.

Thus another experiment (injection of mixture of RVV and TSE) was conducted to see the effect of TSE on the lethal action of RVV. In this study, RVV and TSE were mixed and injected IM into mice. After 24 hours all the mice of the control group (receiving only RVV) died but all mice of the test group (receiving RVV and TSE mixture) survived (Table 1). This findings were in agreement with findings of Ushanandi and group in 2006.

They showed that TSE inhibited PLA_2 enzyme of RVV and neutralized the lethal action of RVV.

All these above findings were based on experiments (injection of mixture of RVV and TSE) and thus another experiment was conducted to find out effects of TSE on the lethal action of RVV, when RVV and TSE were injected separately into different thighs of the mice. It was found that after 24 hours all the mice died (Table 2). However, Ushanandini (2006) had showed tamarind seed extract can neutralize the lethal action of RVV even when the extract was injected 10 minutes after injection of RVV. However, the route of administration in the present study was intramuscular and in Ushanandini's study, the route of administration was intraperitoneal. Rate of absorption of venom from peritoneal cavity was slower than that from intramuscular space. So large amount of venom may be left unabsorbed in the cavity and were inactivated locally by the tamarind seed extract.
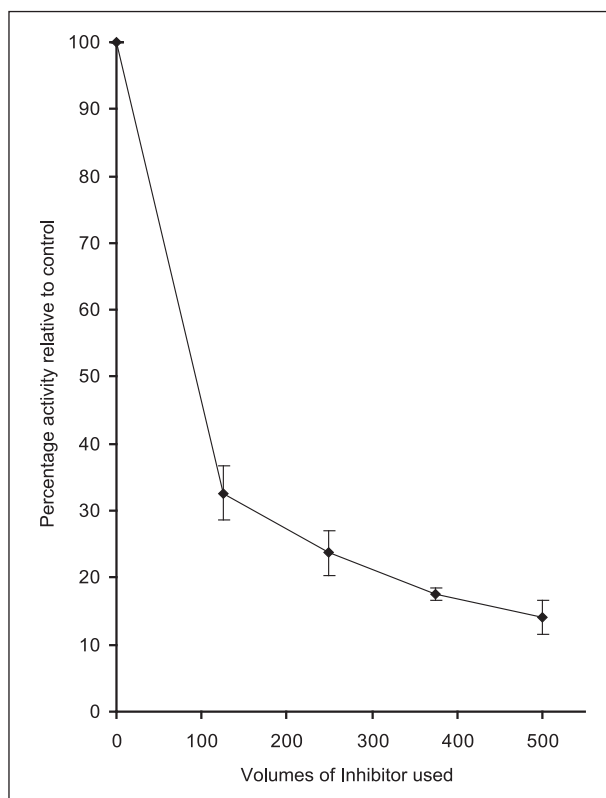
Figure 3. The effect of the tamarind seed extract on caseinolytic enzyme of Russell's viper venom

Table 1. Neutralization potency of Tamarind seed extract (TSE) against lethal action of Russell's viper venom (injection of mixture of RVV and TSE)

	RVV injected	Tamarind seed extract injected (w/w)	No. of animals		Duration of observation period	
			Survived	Dead	Hours	Days
Test	$2LD_{50}$	20 times	5	0	24	7
	$2LD_{50}$	5 times	5	0	24	7
	$2LD_{50}$	1 time	5	0	24	7
Control	$2LD_{50}$	–	0	5	24	–

Table 2. Neutralization potency of Tamarind seed extract (TSE) against lethal action of Russell's viper venom (injecting venom and TSE separately)

	RVV injected	Tamarind seed extract injected (w/w)	No. of animals		Duration of observation period (hrs)
			Survived	Dead	
Test	$2LD_{50}$	20 times	0	5	24
Control	$2LD_{50}$	–	0	5	24

Table 3. Neutralization potency of Tamarind seed extract (TSE) against lethal action of Russell's viper venom *(local infiltration)*

	RVV injected	Tamarind seed extract injected (w/w)	No. of animals		Duration of observation period (hrs)
			Survived	Dead	
Test	$2LD_{50}$	20 times	3	0	24
			2	1	48
			1	2	72
			0	3	96
Control	$2LD_{50}$	–	0	3	24

Thus another experiment (injecting venom and TSE separately) was conducted to find out the effect of TSE on RVV. In this experiment, RVV was injected intramuscularly first and TSE was injected about 30 minutes after it. This time the injection was done near the site of injection of RVV (local infiltration). During 24 hours of observation period, all of the control group died but none of the test group (Table 3). However it was found that one animal died the next day. As the animals were observed continuously, another one died in 72 hrs and last one died in 96 hrs. This seems to suggest that the extract cannot prevent the death even when given within 30 min after injection of RVV but it does prolong the time of survival of experimentally envenomed animals. However it is possible that RVV left unabsorbed in the intramuscular space may react with TSE and was being inactivated. Inactivation may be reversible and venom may become active again which may be the reason why all the mice started dying after 24 hours.

Win Aung and Khin Maung Maung (2012) had showed the effect of local infiltration of anti snake venom serum (ASV) with mice. In their study local infiltration of ASV within one hour after envenomation revealed an obvious neutralization effect. TSE may also play a similar role in the treatment process. Advantage of TSE over ASV is that, supply of ASV is of limited amount, but TSE an extract of plant that grows abundantly in many Asian countries, is of unlimited supply.

Thus in conclusion, TSE can inhibit the action of major enzymes of RVV which causes different clinical effects leading to death. The extract can also neutralize the lethal action if it is mixed with RVV before injection and by local infiltration it can prolong the time of death. From these findings it seems that local infiltration of TSE may be of use with standard treatment with anti-snake venom or as a first aid measure in the treatment of Russell's viper bite patients after conducting a well designed clinical trial.

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