

Preliminary Studies on Antivenom Activity of *Mimosa Pudica* Root Extracts Against Russell's Viper and Saw Scaled Viper Venom By *In Vivo* And *In Vitro* Methods

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Summary

Mimosa pudica aqueous root extracts were tested for their inhibitory activity on various pharmacological effects like lethality, phospholipase activity, edema forming activity, fibrinolytic activity and haemorrhagic activity of Russell's viper and Saw scaled viper venoms. The aqueous extract displayed a significant inhibitory effect on the lethality, phospholipase activity, edema forming activity, fibrinolytic activity and haemorrhagic activity. About 0.13 mg and 0.17 mg of *Mimosa pudica* plant extracts were able to completely neutralize the lethal activity of 2LD₅₀ of *Russell's viper and Saw scaled viper* venoms respectively. The present finding suggests that aqueous extracts of *M. pudica* root possess compounds, which inhibit the activity of *Russell's viper and Saw scaled viper* venoms

Keywords: Venoms, Plant extracts, Lethality, edema, PLA₂

Introduction

Snakebite is a common medical emergency encountered in the tropics and estimated 35,000 to 50,000 people die of snakebite every year in India¹. The common poisonous snakes found in India are Cobra (*Naja naja*), Krait (*Bangarus Caeruleus*), Russell's viper (*Daboia russelli*) and Saw Scaled Viper (*Echis Carinatus*)². Venoms of Russell's viper and Saw-scaled viper of Viperidae family are hemotoxic and mainly affect circulatory system and muscular system causing excessive scarring, hemorrhagic, coagulant defects and hypovolemic shock. The intravenous administration of animal-derived (mostly horse or sheep) antivenoms is the mainstay and the only specific treatment of snake bite envenoming. Anaphylaxis and serum sickness are the major concern with many of the antivenom preparations. These hypersensitivity reactions are mainly caused by the foreign animal proteins present in the antivenom and the probability of a reaction depends partly on the type of antivenom, its manufacturing and concentrating process, and the dose used³. Over the years many attempts have been made for the development of snake venom antagonists especially from plants sources. Several medicinal plants, which appear in old drug recipes or which have been passed on by oral tradition, are believed to be snakebite antidotes. Many Indian medicinal plants are recommended for the treatment of snakebite⁴. *Andrographis paniculata* and *Aristolochia indica* plant extracts possess potent snake venom neutralizing capacity and could be used for therapeutic purposes in case of snakebite envenomations⁵. In almost any part of the world, where venomous snakes occur, numerous plant species are used as folk medicine to treat snakebite. The present investigation explored the Russell's viper and Saw scaled viper venoms neutralizing activity of *Mimosa pudica* plant extracts by *in vivo* and *in vitro* methods.

Materials and Methods

Venom and Experimental animals

The free-dried snake venom powders of Russell's viper and Saw scaled viper were obtained from Irula's Snake Catchers Industrial Co-operative Society Limited, Chennai and was stored at 4°C. Male inbred Swiss albino mice 18-20 gm were used for efficacy studies. Institutional Animal Ethics Committee clearance at Institute of vector control and Zoonoses, Hosur, was obtained to conduct the experiment.

Medicinal Plants and Preparation of Extracts

Mimosa pudica plants were collected from Nehru Gardens of Nehru Arts and Science college. The extraction was carried out by the method of Uhegbu *et al.* (2005)⁶ using distilled water as the solvent. 20 g of powdered sample of the herb was extracted by soaking in 180 mL of distilled water in a beaker, stirred for about 6 minutes and left overnight. Thereafter, the solution was filtered using filter paper (Whatman No. A-1) and the extracts were evaporated to dryness under reduced pressure below 40°C. The plant extracts were expressed in terms of dry weight.

Pharmacological characterization of venom and its neutralization by *Mimosa pudica* root extracts by *in vivo* and *in vitro* methods

Lethal toxicity

The median lethal dose (LD₅₀) of Russell's viper and Saw scaled viper venom was determined according to the method developed by Theakston and Reid 1983⁷. Various doses of venom in 0.2 ml of physiological saline was injected into the tail vein of mice (18-20gms), using groups of 3-5 mice at each venom dose. The LD₅₀ was calculated with the confidence limit at 50% probability by the analysis of deaths occurring within 24 h of venom injection. The anti-lethal potentials of *Mimosa pudica* plant extracts were determined against 2LD₅₀ of Russell's viper and Saw scaled viper venom. Various amount of Plant extracts (μl) were mixed with 2LD₅₀ of venom sample and incubated at 37°C for 30 minutes and then injected intravenously into mice. 3 -5 mice were used at each antivenom dose. Control mice received same amount of venom without antivenom (Plant extracts). The median Effective Dose (ED₅₀) calculated from the number of deaths within 24h of injection of the venom/antivenom mixture. The ED₅₀ was expressed as μl antivenom/mouse and calculated by probit analysis.

Edema- forming Activity

The Minimum edema-forming dose (MED) of Russell's viper and Saw scaled viper venom was determined by the method of Lomonte *et al.* (1993)⁸ and Camey *et al.* (2002)⁹. The Minimum edema-forming dose (MED) was defined as the least amount of venom which when injected subcutaneously into mice footpad results in 30% edema with in 6 hours of venom injection. The thickness of each footpad was measured every 30 min after venom injection with a low-pressure spring caliper. The ability of *Mimosa pudica* plant extract in neutralizing the Edema- forming activity were carried out by pre-incubating the constant amount of venom and various dilutions *Mimosa pudica* plant extracts and incubated for 30 minutes at

37°C. Then, groups of four mice (18 - 20g) were injected subcutaneously in the right footpad with 50µl of the mixtures, containing venom/plant extracts, whereas the left footpad received 50µl of PBS alone. Control mice were injected with venom in the right footpad and 50µl of PBS in the left footpad. 1 hour after injection edema was evaluated as described by Yamakawa *et al.*, 1976¹⁰. Edema was expressed as the percentage increase in thickness of the right footpad compared to the right footpad of the control mice.

Haemorrhagic activity

The minimum haemorrhagic dose (MHD) of Russell's viper and Saw scaled viper venom was determined by the method described by Theakston and Reid, 1983⁷. The minimum haemorrhagic dose was defined as the least amount of venom which when injected intradermally (i.d.) into mice results in a haemorrhagic lesion of 10mm diameter in 24 hours. Neutralization of the haemorrhagic activity was estimated by mixing a fixed amount of venom with different amounts plant extracts. The Plant extract-venom mixture was incubated at 37°C for 1 h and 0.1 ml of the mixture was injected intradermally into mice. The haemorrhagic lesion was estimated after 24 h.

Phospholipase activity

Phospholipase A2 activity was measured using an indirect hemolytic assay on agarose-erythrocyte-egg yolk gel plate by the methods described by Gutierrez *et al.*, 1988¹¹. Increasing doses of Russell's viper and Saw scaled viper venom (µg) was added to 3mm wells in agarose gels (0.8% in PBS, pH 8.1) containing 1.2% sheep erythrocytes, 1.2% egg yolk as a source of lecithin and 10mM CaCl₂. Slides were incubated at 37°C overnight and the diameters of the hemolytic halos were measured. Control wells contained 15µl of saline. The minimum indirect hemolytic dose (MIHD) corresponds to a dosage of venom, which produced a hemolytic halo of 11mm diameter. The efficacy of *Mimosa pudica* Plant extract in neutralizing the phospholipase activity was carried out by mixing constant amount of venom (µg) with different amount of plant extract (µl) and incubated for 30 minutes at 37°C. Then, aliquots of 10µl of the mixtures were added to wells in agarose-egg yolk-sheep erythrocyte gels. Control samples contain venom without Plant extracts. Plates were incubated at 37°C for 20 hours. Neutralization expressed as the ratio mg antibodies/mg venom able to reduce by 50% the diameter of the hemolytic halo when compared to the effect induced by venom alone.

Procoagulant activity

The procoagulant activity was done according to the method described by Theakston and Reid, 1983⁷ modified by Laing *et al.*, 1992¹². Various amounts of venom dissolved in 100µl PBS (pH 7.2) was added to human citrated plasma at 37°C. Coagulation time was recorded and the Minimum Coagulant Dose (MCD) was determined as the venom dose, which induced clotting of plasma within 60 seconds. Plasma incubated with PBS alone served as control. In neutralization assays Constant amount of venom was mixed with various dilutions of *Mimosa pudica* plant extracts. The mixtures were incubated for 30 minutes at 37°C. Then 0.1ml of mixture was added to 0.3ml of citrated plasma and the clotting times recorded. In control tubes plasma was incubated with either venom alone or plant extracts alone.

Neutralization was expressed as effective dose (ED), defined as the ratio μl antivenom (plant extracts)/mg venom at which the clotting time increased three times when compared with clotting time of plasma incubated with two MCD of venom alone.

Fibrinolytic activity

A modified plaque assay was used (Rojas et al., 1987) ¹³. The minimum fibrinolytic concentration was defined as the concentration of venom that induced a fibrinolytic halo of 10mm diameter. Neutralization experiments were performed by incubating a constant amount of venom with varying amount of *Mimosa pudica* plant extracts at 37°C for 1 h. After incubation, the mixture was applied to the wells in the plaque. After 18 h of incubation at 37°C, fibrinolytic halos were measured.

Statistical Analysis

Statistical evaluation was performed using XL stat 2007 and SPSS 10 Softwares. $P < 0.005$ was considered statistically significant.

Results

The antivenom potential of *Mimosa pudica* plant extract was tested against Russell's viper and Saw scaled viper venom by in vivo and in vitro methods. The lethal toxicity (LD₅₀) of Russell's viper and Saw scaled viper venom was assessed using 18g, Balb/c strain mice. About 8 μg of Russell's viper and 12 μg of Saw scaled viper venom was found to be LD₅₀ for 18g of mice. The neutralization of lethality was done by mixing constant amount of venom (2LD₅₀) with various dilutions of *Mimosa pudica* Plant extracts and incubated at 37°C for 30 minutes prior to injection. We found that 0.13 mg and 0.17 mg of *Mimosa pudica* plant extracts were able to completely neutralize the lethal activity of 2LD₅₀ of Russell's viper and Saw scaled viper venom respectively (Table 1).

Table 1: Neutralization of Russell's viper and Saw scaled viper venom induced lethality by *Mimosa pudica* Plant extracts

Venoms	Dose of venom (μg)	Neutralization of venom by <i>Mimosa pudica</i> root extracts (ED ₅₀ in mg)
Russell's viper	16 (2LD ₅₀)	0.13 mg
Saw scaled viper	24 (2LD ₅₀)	0.17 mg

In edema forming activity, the mice immunized with Russell's viper and Saw scaled viper venoms showed increase in footpad thickness. About 5 μg of Russell's viper and 8 μg of Saw scaled viper venom induced edema formation within 3h which is considered as 100% activity. The edema was reduced up to 30% when 2.7 mg of plant extract per mg venom was given. There was no further reduction in the percentage of edema even when there was an increase in antivenom dose (Fig.1).

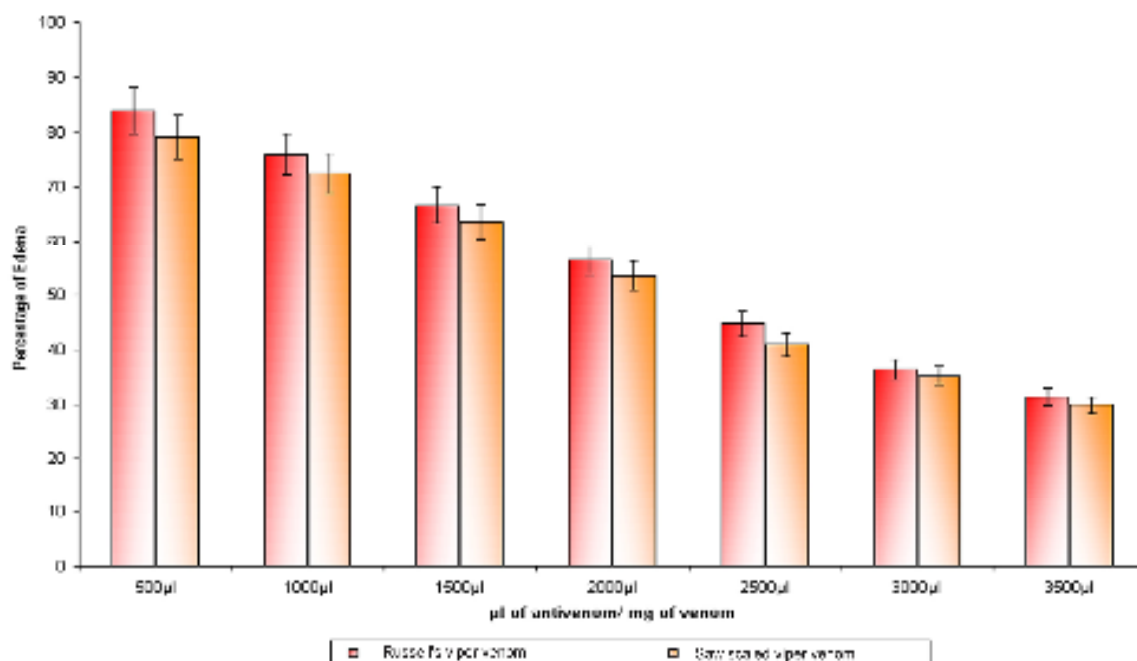


Fig 1- Neutralization of edema induced by Russell's viper and Saw scaled viper venom by *Mimosa pudica* plant extracts in experiments with pre-incubation. Various mixtures of Russell's viper and Saw scaled viper venom and *Mimosa pudica* plant extracts were incubated and tested in the footpad assay. Edema was assessed 1 h after injection and expressed as percentage. Edema induced in control mice (venom alone) was considered as 100% activity. *Mimosa pudica* plant extracts inhibited the Edema forming activity and edema was reduced upto 30%. $P < 0.005$ at all antivenoms/ venom ratios.

In the case of hemorrhagic activity, only Saw scaled viper venom produced visible hemorrhagic spot. About 8 μg of venom produced a hemorrhagic spot of 10mm diameter (Minimum Haemorrhagic Dose). We found that 1.5 mg of *Mimosa pudica* Plant extracts was completely neutralize hemorrhagic activity produced by Saw scaled viper venom. In phospholipase activity (PLA₂), Russell's viper and Saw scaled viper venom able to produce hemolytic haloes in agarose-sheep erythrocytes gels. About 15 μg of Russell's viper and 10 μg of Saw scaled viper venom produced 11mm diameter hemolytic halo, which is considered to be 1U (U/10μg). This shows that Russell's viper and Saw scaled viper venoms have the enzymes (PLA₂) that has the ability to lyse sheep RBC's. *Mimosa pudica* plant extracts were capable of inhibiting PLA₂ dependent hemolysis of sheep RBC's induced by Russell's viper and Saw scaled viper venom in a dose dependent manner. We found that that 0.12 mg and 0.14 mg of *Mimosa pudica* plant extracts were able to completely inhibit PLA₂ dependent hemolysis of sheep RBC's induced by Russell's viper and Saw scaled viper venom respectively (Table 2).

Table 2: Phospholipase activity of Russell's viper and Saw scaled viper venom and its neutralization by *Mimosa pudica* Plant extracts

Venoms	Dose of venom (μg)	Neutralization of venom by <i>Mimosa pudica</i> root extracts (ED ₅₀ in mg)
<i>Russell's viper</i>	15 (1 Unit)	0.12 mg
<i>Saw scaled viper</i>	10 (1 Unit)	0.14 mg

The minimum coagulant dose (MCD) was determined as the venom dose inducing clotting of plasma in 60s. About 120 μg of Russell's viper and Saw scaled viper venom clotted human citrated plasma within 60s. In the neutralization assay, the absence of clot formation shows the neutralizing ability of both Plant extracts. We found that that 1.4 mg of *Mimosa pudica* plant extracts were able to completely neutralize coagulant activity. High dose of venom caused rapid clotting that required very high dose of antivenom to neutralize. The fibrinolytic effect was effectively antagonized by the *Mimosa pudica* plant extract. The ED₅₀ of *Mimosa pudica* were found to be 0.7 and 0.9 mg for Russell's viper and Saw scaled viper respectively.

Discussion

Snakebites being a major public health problem claim a large number of lives in the Indian subcontinent. Antisnake venom remains the specific antidote for snake venom poisoning. This antisnake venom are usually derived from horse sera. They contain horse immunoglobulins, which frequently caused complement mediated side effects, and other proteins that cause serum sickness and occasionally, anaphylactic shock. Although, use of plants against the effects of snakes bite has been long recognized, more scientific attention has been given since last 20 years¹⁴. Many Indian medicinal plants are recommended for the treatment of snakebites⁴. *Andrographis paniculata* and *Aristolochia indica* plant extracts possess potent snake venom neutralizing capacity and could be used for therapeutic purposes in case of snakebite envenomations⁵. In our present study we check the antivenom potential of *Mimosa pudica* plant extracts against Russell's viper and Saw scaled viper venom. Various pharmacological activities like lethality, edema forming activity, hemorrhagic activity, fibrinolytic activity, phospholipase activity (PLA₂), procoagulant activity caused by Russell's viper and Saw scaled viper venom were carried out. Neutralization of these pharmacological effects was carried out using *Mimosa pudica* plant extract. Neutralization studies can be performed by incubating of venom and plant extract prior to testing (pre-incubation method). The results showed that the *Mimosa pudica* plant extract was capable of neutralizing the lethality induced by the venom. *Mimosa pudica* plant extract was capable of inhibiting PLA₂ dependent hemolysis of sheep RBCs in a dose dependent manner. Edema-forming activity was assessed for Russell's viper and Saw scaled viper venom and *Mimosa pudica* plant extract was found to be effective in neutralization of edema induced by venoms. There was a significant decrease in the edema (footpad thickness) when there was an increase in the antivenom (plant extract) dose.

Procoagulant activity induced by Russell's viper and Saw scaled viper venom was studied using human citrated plasma and *Mimosa pudica* plant extract was found to be effective in the neutralization of procoagulant activity. *Mimosa pudica* plant extract was effectively antagonised the fibrinolytic activity. The main conclusion of this study is that the present experimental results indicate that *Mimosa pudica* plant extract was effective in neutralizing the main toxic effects of the Russell's viper and Saw scaled viper venoms. The potency of the antivenom antibodies can still increase with the use of highly purified plant extracts.

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