

Antitoxin activity of *Mucuna pruriens* aqueous extracts against Cobra and Krait venom by in vivo and in vitro methods

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Abstract: Aqueous extracts of *Mucuna pruriens* seeds were tested for their activity on various pharmacological effects like lethality, phospholipase activity, edema forming activity, fibrinolytic activity and haemorrhagic activity of cobra and krait venoms. The aqueous extract displayed a significant inhibitory effect on the lethality, phospholipase activity, edema forming activity, fibrinolytic activity and haemorrhagic activity. About 0.16 mg and 0.19 mg of *Mucuna pruriens* seed extracts were able to completely neutralize the lethal activity of 2LD₅₀ of cobra and krait venom respectively. The present finding suggests that aqueous extracts of *Mucuna pruriens* seeds possess compounds, which inhibit the activity of cobra and krait venoms.

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

1. 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. The venoms of Cobra and Krait of Elapidae family are neurotoxic in nature, considered to attack victim's central nervous system and usually results in heart failure. Antivenom immunotherapy is the only specific treatment against snake venom envenomation. There are various side effects of antivenom such as anaphylactic shock, pyrogen reaction and serum sickness. Most of these symptoms may be due to the action of high concentrations of nonimmunoglobulin proteins present in commercially available hyper immune antivenom³. Over the years many attempts have been made for the development of snake venom antagonists especially from plants sources. 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. The aqueous extracts of *Mucuna pruriens* possess compounds, which inhibit the activity of Cobra and Krait venoms⁶. 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 Cobra and Krait venom neutralizing activity of *Mucuna pruriens* plant extracts by in vivo and in vitro methods.

2. Materials and Methods

2.1 Venom and Experimental animals

The free-dried snake venom powders of Cobra and Krait 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 g were used for efficacy studies. Institutional Animal Ethics Committee clearance at Institute of

vector control and Zoonoses, Hosur, was obtained to conduct the experiment.

2.2 Medicinal Plants and Preparation of Extracts

Mucuna pruriens 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)⁷ using distilled water as the solvent. 20 g of powdered sample of the seed 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.

2.3 Pharmacological characterization of venom and its neutralization by *Mucuna pruriens* seed extracts by in vivo and in vitro methods

2.3.1 Lethal toxicity

The median lethal dose (LD₅₀) of Cobra and Krait venom was determined according to the method developed by Theakston and Reid 1983⁸. Various doses of venom in 0.2 mL of physiological saline was injected into the tail vein of mice (18-20 g), 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 *Mucuna pruriens* seed extracts were determined against 2LD₅₀ of Cobra and Krait venom. Various amount of seed extracts (μL) were mixed with 2LD₅₀ of venom sample and incubated at 37°C for 30 min 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 24 h of injection of the venom/antivenom mixture. The ED₅₀ was expressed as μL antivenom/mouse and calculated by probit analysis.

2.3.2 Edema- forming Activity

The Minimum edema-forming dose (MED) of Cobra and Krait venom was determined by the method of Lomonte et al. (1993)⁹ and Camey et al. (2002)¹⁰. 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 within 6 h of venom injection. The thickness of each footpad was measured every 30 min after venom injection with a low-pressure spring caliper¹¹. The ability of *Mucuna pruriens* seed extract in neutralizing the Edema- forming activity were carried out by pre-incubating the constant amount of venom and various dilutions *Mucuna pruriens* seed extracts and incubated for 30 min at 37°C. Then, groups of four mice (18 – 20 g) were injected subcutaneously in the right footpad

with 50 μL of the mixtures, containing venom/plant extracts, whereas the left footpad received 50 μL of PBS alone. Control mice were injected with venom in the right footpad and 50 μL of PBS in the left footpad. 1 h after injection edema was evaluated as described by Yamakawa et al. 1976¹². Edema was expressed as the percentage increase in thickness of the right footpad compared to the right footpad of the control mice.

2.3.3 Haemorrhagic activity

The minimum haemorrhagic dose (MHD) of Cobra and Krait venom was determined by the method described by Theakston and Reid, 1983. 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 10 mm diameter in 24 h. Neutralization of the haemorrhagic activity was estimated by mixing a fixed amount of venom with different amounts seed extracts. The seed 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.

2.3.4 Phospholipase activity

Phospholipase A2 activity was measured using an indirect hemolytic assay on agarose-erythrocyte-egg yolk gel plate by the method described by Gutierrez et al. 1988¹³. Increasing doses of Cobra and Krait venom (μg) was added to 3 mm 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 10 mM CaCl₂. Slides were incubated at 37°C overnight and the diameters of the hemolytic halos were measured. Control wells contained 15 μL of saline. The minimum indirect hemolytic dose (MIHD) corresponds to a dosage of venom, which produced a hemolytic halo of 11 mm diameter. The efficacy of *Mucuna pruriens* seed extract in neutralizing the phospholipase activity was carried out by mixing constant amount of venom (μg) with different amount of seed extract (μL) and incubated for 30 min at 37°C. Then, aliquots of 10 μL of the mixtures were added to wells in agarose-egg yolk-sheep erythrocyte gels. Control samples contain venom without extracts. Plates were incubated at 37°C for 20 h. 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.

2.3.5 Procoagulant activity

The procoagulant activity was done according to the method described by Theakston and Reid, 1983 modified by Laing et al. 1992¹⁴. Various amounts of venom dissolved in 100 μL 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 sec. Plasma incubated with PBS alone served as control. In neutralization assays Constant amount of venom was mixed with various dilutions of *Mucuna pruriens* seed extracts. The mixtures were incubated for 30 min at 37°C. Then 0.1 mL of mixture was added to 0.3 mL 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 (seed 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.

2.3.6 Fibrinolytic activity

A modified plaque assay was used¹⁵. The minimum fibrinolytic concentration was defined as the concentration of venom that induced a fibrinolytic halo of 10 mm diameter. Neutralization experiments were performed by incubating a constant amount of venom with varying amount of *Mucuna pruriens* seed 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.

2.4 Statistical Analysis

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

3. Results

The antivenom potential of *Mucuna pruriens* plant extract was tested against Cobra and Krait venom by in vivo and in vitro methods. The lethal toxicity (LD_{50}) of Cobra and Krait venom was assessed using 18 g, Balb/c strain mice. About 10 μg of Cobra and 3 μg of Krait venom was found to be LD_{50} for 18 g of mice. The neutralization of lethality was done by mixing constant amount of venom (2LD_{50}) with various dilutions of *Mucuna pruriens* Plant extracts and incubated at 37°C for 30 min prior to injection. We found that 0.16 mg and 0.19 mg of *Mucuna pruriens* plant extracts were able to completely neutralize the lethal activity of 2LD_{50} of Cobra and Krait venom respectively (Table 1, Fig1).

In edema forming activity, the mice immunized with Cobra and Krait venoms showed increase in footpad thickness. About 7 μg of cobra and 2 μg of Krait venom induced edema formation within 3h which is considered as 100% activity. The edema was reduced up to 30% when 2.8 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. In the case of hemorrhagic activity,

Cobra and Krait venom did not produced any visible hemorrhagic spot. In phospholipase activity (PLA_2), Cobra and Krait venom able to produce hemolytic haloes in agarose-sheep erythrocytes gels. About 15 μg of Cobra and 10 μg of Krait venom produced 11mm diameter hemolytic halo, which is considered to be 1U ($\text{U}/10 \mu\text{g}$). This shows that Cobra and Krait venoms have the enzymes (PLA_2) that has the ability to lyse sheep RBC's. *Mucuna pruriens* extracts were capable of inhibiting PLA_2 dependent hemolysis of sheep RBC's induced by Cobra and Krait venom in a dose dependent manner. We found that that 0.15 mg and 0.18 mg of *Mucuna pruriens* plant extracts were able to completely inhibit PLA_2 dependent hemolysis of sheep RBC's induced by Cobra and Krait venom respectively.

The minimum coagulant dose (MCD) was determined as the venom dose inducing clotting of plasma in 60 sec. About 40 μg of Cobra and Krait venom clotted human citrated plasma within 60 sec. In the neutralization assay, the absence of clot formation shows the neutralizing ability of both Plant extracts. We found that that 1.8 mg of *Mucuna pruriens* plant extracts were able to completely neutralize coagulant activity. The fibrinolytic effect was effectively antagonized by the *Mucuna pruriens* seed extract. The ED_{50} of *Mucuna pruriens* were found to be 0.9 and 1.2 mg for Cobra and Krait respectively.

4. 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 years. Many Indian medicinal plants are recommended for the treatment of snakebites⁴. In our present study we check the antivenom potential of *Mucuna pruriens* plant extracts against Cobra and Krait venom. Various pharmacological activities like lethality, edema forming activity, hemorrhagic activity, fibrinolytic activity, phospholipase activity (PLA_2), procoagulant activity caused by Cobra and Krait venom were carried out. Neutralization of these pharmacological effects was carried out using *Mucuna pruriens* 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 *Mucuna pruriens* plant extract was capable of neutralizing the lethality induced by the venom. The Cobra and Krait venom showed the presence of PLA_2

enzymes by means of producing hemolytic haloes in indirect hemolytic assays. *Mucuna pruriens* plant extract was capable of inhibiting PLA2 dependent hemolysis of sheep RBCs in a dose dependent manner. Edema-forming activity was assessed for Cobra and Krait venom and *Mucuna pruriens* seed 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 Cobra and Krait venom was studied using human citrated plasma and *Mucuna pruriens* seed extract was found to be effective in the neutralization of procoagulant activity. *Mucuna pruriens* seed extract was effectively antagonised the fibrinolytic activity. The main conclusion of this study is that the present experimental results indicate that *Mucuna pruriens* seed extract was effective in neutralizing the main toxic effects of the Cobra and Krait venoms.

Table 1: Neutralization of Cobra and Krait venom induced lethality by *Mucuna pruriens* seed extracts

Venoms	Dose of venom (µg)	Neutralization of venom by <i>Mucuna pruriens</i> extracts (ED ₅₀ in mg)
<i>Cobra</i>	20 (2LD ₅₀)	0.16 mg
<i>Krait</i>	6 (2LD ₅₀)	0.19 mg

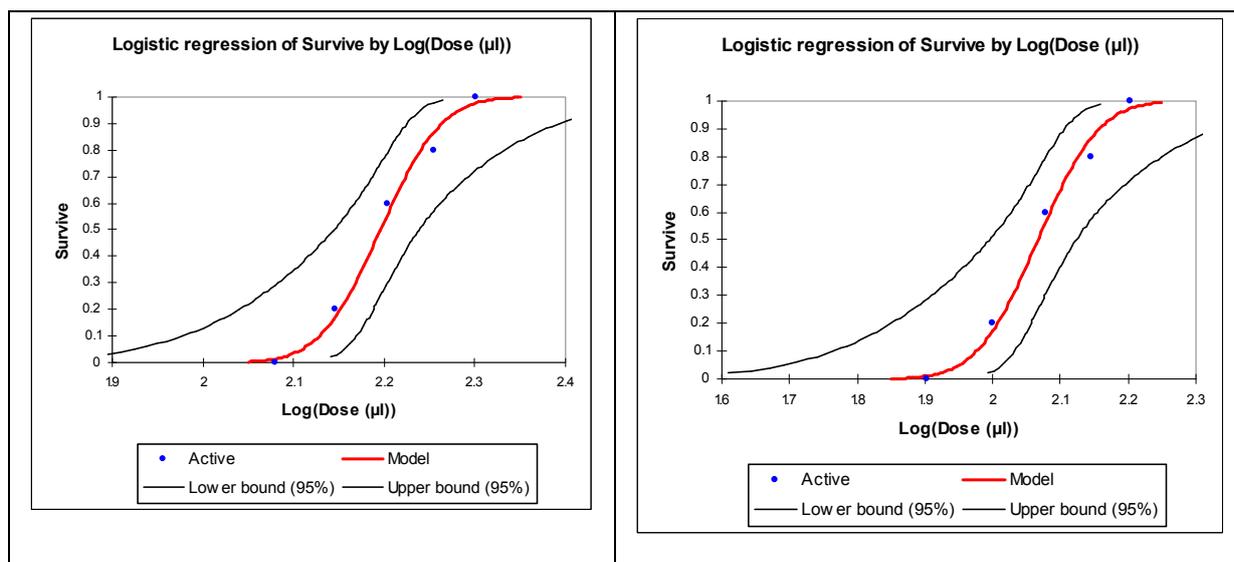


Fig 1 - Dose response curve for Neutralization of Lethality by *Mucuna pruriens* plant extracts against Cobra and Krait venom in experiments involving preincubation of venom (2 x LD₅₀) and various concentrations of Plant extracts. The median effective dose for Cobra was 157.0µl (139.45 – 173.16) and Krait was 119.58µl (98.80 – 132.73). P < 0.005.

Table 2: Phospholipase activity of Cobra and Krait venom and its neutralization by *Mucuna pruriens* seed extracts

Venoms	Dose of venom (µg)	Neutralization of venom by <i>Mucuna pruriens</i> extracts (ED ₅₀ in mg)
<i>Cobra</i>	15 (1 Unit)	0.15 mg
<i>Krait</i>	10 (1 Unit)	0.18 mg

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