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In vivo and *In vitro* Neutralizing Potential of *Rauvolfia serpentina* Plant Extract Against *Daboia russelli* Venom

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Abstract: *Snake venom* neutralizing potential of *Rauvolfia serpentina* plant extract was tested by *in vitro* and *in vivo* methods against *Daboia russelli* venom. The *in vivo* assessment of venom lethality (LD_{50}) of *Daboia russelli* venom was found to be 0.628 µg/g. *Rauvolfia serpentina* plant extract was effectively neutralized the venom lethality and effective dose (ED_{50}) was found to be 10.99 mg/ $3LD_{50}$ of venom. Various *in vitro* neutralization tests like Procoagulant, Direct and Indirect haemolytic activities of *Daboia russelli* venom were carried out and *Rauvolfia serpentina* plant extract was effectively neutralize all the toxic effects induced by the venom. In Acute Oral Toxicity all animals survived and appeared active and healthy throughout the study. There were no signs of gross toxicity, adverse pharmacological effects or abnormal behavior. The LD_{50} of *Rauvolfia serpentina* plant extract was >2000 mg/kg. These findings confirmed that *Rauvolfia serpentina* plant extract possess some compounds which inhibit the toxins present in *Daboia russelli* venom.

Key words: *Daboia russelli* Venom · Plant Extract · Lethality · PLA_2

INTRODUCTION

Snake bite envenomations is a serious health problems particularly in rural areas due to lack of basic health facilities and transportation. Because of transportation many victims fail to reach hospital in time, some even die before reaching hospital. Though there are varieties of snakes in India, only four among them are venomous. These are the Cobra (*Naja naja*), Krait (*Bangarus caeruleus*), Russell's viper (*Daboia russelli*) and Saw Scaled Viper (*Echis Carinatus*) [1]. Snake bites are seen often among agricultural workers and among those going to the forest. Coimbatore city is surrounded by forests and harbours many snakes. A report in daily news paper (Times of India) quoted that at least 100 snake bite cases are reported every month at Coimbatore Medical College Hospital (CMCH) and most causalities happen when the snake is a Russell's Viper, Common Krait or Cobra. Most often, snake bites occur during the rainy season. While 4 percent of the cases are fatal, 10 percent suffer serious health complications. Coimbatore Medical College Hospital alone handled an alarming 202 cases of snake bites during August and September 2012 [2]. At present polyvalent Antivenom, a type of serum is the only life saving choice for snake bite envenomations. But due to their cost and various side effects it is important to find an alternative remedy to treat snake bite envenomations. Over the years many attempts have been made for the development of antidote for snake venom especially from plants sources. Extracts from plants have been used among traditional healers, especially in tropical areas where here are plentiful sources, as therapy for snakebite for a long time. India has a rich tradition of the usage of medicinal plants and various herbal plants are likely to be snakebite antidotes and are recommended for the treatment of snakebite envenomations [3]. Methanolic extracts of *Andrographis paniculata* and *Aristolochia indica* plant extracts possess potent snake venom neutralizing capacity and could potentially be used for therapeutic purposes in case of snakebite

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envenomation [4]. *A. paniculata, C. magna, G. superba and H. javanica* plant extracts contains compounds which can able used in case of snakebite envenomation, especially against the local effects of cobra venom [5]. The present investigation explored the *in vitro* and *in vivo* neutralizing potential of *Rauvolfia serpentina* extract against *Daboia russelli* venom.

MATERIALS AND METHODS

Venom and Experimental Animals: The freeze-dried snake venom powder of *Daboia russelli* was obtained from Haffkine Institute for Training, Research and Testing, Mumbai and was stored at 4°C. *In vivo* tests were carried out in J.S.S. College of Pharmacy, Ootacamund, Tamil Nadu (Ethics committee approval Number: JSSCP/IAEC/CADRAT/07/2013 dated 29.04.2013).

Medicinal Plans and Preparation of Extracts: *Rauvolfia serpentina* plant was obtained from Arya Vaidya Pharmacy, Coimbatore and the extract was prepared 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 min and left overnight. Thereafter, the solution was filtered using filter paper (Whatman No. 1) and the extracts were evaporated to dryness under reduced pressure below 40°C. The plant extracts were expressed in terms of dry weight [6].

Acute Oral Toxicity: Acute oral toxicity of all the *Rauvolfia serpentina* plant extract was performed as per OECD guidelines 423. A limit test at 2000 mg/kg body weight of the extract was administered. Briefly, two thousand milligrams of the test substance per kilogram of body weight was administered to 3 healthy mice by oral gavages. The animals were observed for mortality, signs of gross toxicity and behavioral changes at least once daily for 14 days. Body weights were recorded prior to administration and again on Days 7 and 14 (day of termination). Necropsies were performed on all animals at terminal sacrifice.

***In vivo* Assessment of Venom Toxicity (LD_{50})**

LD_{50} Range-Finding Test: Various venom doses were prepared using saline solution as diluent and aliquots of a precise volume (0.2–0.5 ml) of each dose were injected, using one mouse per dose, by the intravenous route, in the tail vein. Deaths were recorded at 24 hours. On the basis of this preliminary dose-finding experiment, a range of venom doses causing 0% to 100% lethality was established and thus narrowed the range of venom doses required to formally estimate the toxic activity of the venom.

The Median Lethal Dose (LD_{50}) Assay: Groups of 5–6 mice of a defined weight range were injected intravenously, in the tail vein, with a precise volume (0.2–0.5 ml) of solutions of varying doses of venom dissolved in sterile saline solution. A minimum of 5 mice is the smallest number recommended for obtaining a statistically significant result. Deaths were recorded at 24 hours and LD_{50} was estimated by Probit analysis. One venom LD_{50} is defined as the minimal amount of venom causing death in 50% of the mice [7, 8].

Neutralization of Lethality (ED_{50})

ED_{50} Range-finding Test: The selected multiple of the venom LD_{50} ($3LD_{50}$) was mixed with different doses of plant extract and incubated at 37°C for 30 minutes and each mixture injected into a single mouse. This preliminary test established a range of antivenom volumes that result in 100% survival and 100% death of the injected mice and thus narrows down the range of doses required for the formal ED_{50} test.

The Median Effective Dose (ED_{50}) Assay: This test involved the incubation of a fixed amount of venom ("challenge dose", usually corresponding to $3LD_{50}$), with various volumes of the antivenom (Plant extract) adjusted to a constant final volume with saline solution. The mixtures were incubated for 30 minutes at 37°C and then aliquots of a precise volume (0.2–0.5 ml) of each mixture were injected into groups of generally 5 or 6mice of a defined weight range by the intravenous route, using the tail vein. A control group was injected with a mixture of the venom "challenge dose" with saline solution alone (no antivenom) to confirm that the venom "challenge dose" induced 100% lethality. Centrifugation of the antivenom– venom mixtures is not recommended because residual venom toxicity may remain in the immunoprecipitates. After injection, deaths were recorded at 24 hours and the results were analysed using Probit analysis (Miller and Tainter, 1944).The median effective dose (ED_{50}) of an antivenom is defined as the volume of antivenom that protects 50% of the mice injected.

In vitro Assessment of Venom Toxicity and Neutralization Potential of *Rauvolfia serpentina* Extracts Against *Daboia russelli* Venom

Direct Hemolysis Assay: The hemolytic action of venom and plant extract was studied *in vitro* by using RBC. Briefly, 5ml of citrated blood was centrifuged for 10minutes at 900 rpm. The supernatant was poured off and the pellet was washed twice with physiological salt solution. Control tubes consist of 5ml of physiological saline and 0.5ml of RBC mixture and for 100% hemolysis 5ml of distilled water mixed with 0.5ml of washed RBC. Experimental sample contains 5ml of venom/extract and 0.5ml of washed RBC. The tubes were put in a thermostat for 1hr at 37°C and centrifuged at 2000rpm for 20mts. The supernatant fluid was poured off to separate tubes to measure the optical density using spectrophotometer at a wave length of 540nm against water. The calculation of hemolysis was done by the formula.

$$\frac{\text{Experimental sample} - \text{Control sample}}{\text{100\% hemolysis}} \times 100$$

Indirect Hemolysis Assay (PLA_2 activity): Phospholipase A_2 activity was measured using an indirect hemolytic assay on agarose–erythrocyte–egg yolk gel plate by the methods described by Gutierrez *et al.* [9]. Increasing concentrations of *Daboia russelli* 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 $CaCl_2$. 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 concentration of venom, which produced a hemolytic halo of 11mm diameter. The efficacy of *Rauvolfia serpentina* 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 extract. Plates were incubated at 37°C for 20 hours. Neutralization expressed as the ratio mg plant extract/mg venom able to reduce by 50% the diameter of the hemolytic halo when compared to the effect induced by venom alone.

Procoagulant Activity: 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 concentration, 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 plant extract. 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 extract alone. Neutralization was expressed as effective dose (ED), defined as the ratio µl antivenom (plant extract)/mg venom at which the clotting time increased three times when compared with clotting time of plasma incubated with two MCD of venom alone [10, 11].

RESULTS

Antivenom potential of *Rauvolfia serpentina extract against Daboia russelli* venom was studied by *in vivo* and *in vitro* methods. *In vivo* assessment of venom toxicity (LD_{50}) of *Daboia russelli* venom was assessed by LD_{50} range-finding test and the median lethal dose (LD_{50}) assay using mice (18–20 g). LD_{50} of *Daboia russelli* venom was calculated by Miller and Tainter method and we was found to be 0.628 µg/g . (Table 1 and Fig 1). Venom-neutralizing potency test (ED_{50}) using *Rauvolfia serpentina* extract was carried out by preincubating constant amount of venom ($3LD_{50}$) with various dilutions of *Rauvolfia serpentina* extracts prior to injection. Calculation of ED_{50} of *Rauvolfia serpentina* against $3LD_{50}$ of venom was done by Miller and Tainter method and found to be 10.99 mg / $3LD_{50}$ venom (Table 2 and Fig 2). In Acute Oral Toxicity all animals survived and appeared active and healthy throughout the study. There were no signs of gross toxicity, adverse pharmacological effects or abnormal behavior. Gross necropsy findings at terminal sacrifice were unremarkable. Based on the above findings, the LD_{50} of *Rauvolfia serpentina* plant extract was >2000 mg/kg. Direct hemolysis of *Daboia russelli* venom produced 89.55% hemolysis and *Rauvolfia serpentina* extract can able to neutralize the hemolysis of RBC's produced by *Daboia russelli* venom up to 35%. In phospholipase activity (PLA2) 8 µg of *Daboia russelli* venom was able to produce 11mm diameter hemolytic halo, which is considered to be 1Unit. *Rauvolfia serpentina* extract can capable of inhibiting PLA_2 dependent hemolysis of sheep RBC's induced by *Daboia russelli* venom in a dose dependent manner. In procoagulant activity 120µg of *Daboia russelli* venom can able to clot human citrated plasma in 60 seconds. In neutralization test absence of clot formation showed the neutralizing ability of *Rauvolfia serpentina* extract.

Table 1: Death Percentage of mice receiving various doses of *Daboia russelli* venom by Miller and Tainter method (n=5).

Dose (µg/g)	Adjusted (Dose×100)	Log dose	Death/Total	Dead%	Corrected formula%	Probit values
0.05	5	0.7	0/5	0	5	3.36
0.1	10	1	1/5	20	20	4.16
0.25	25	1.4	2/5	40	40	4.75
0.5	50	1.7	2/5	40	40	4.75
1.0	100	2.0	3/5	60	60	5.25
2.5	250	2.4	3/5	60	60	5.25
5.0	500	2.7	5/5	100	95	6.64

Corrected formula: For the 0% dead: 100(0.25/n) = 100(0.25/5) = 5

For the 100% dead: 100[(n-0.25)/n] = 100[(5-0.25)/5] = 95, n is the number of animals in the group

Table 2: Death Percentage of mice receiving various doses of *Rauvolfia serpentina* against $3LD_{50}$ of *Daboia russelli* venom by Miller and Tainter method (n=5)

Dose (mg/100µL)	Adjusted (Dose×100)	Log dose	Survival/Total	Dead%	Corrected formula%	Probit values
1	100	2	0/5	0	5	3.36
2.5	250	2.4	0/5	0	5	3.36
5	500	2.7	1/5	20	20	4.16
10	1000	3	2/5	40	40	4.75
20	2000	3.3	3/5	60	60	5.25
40	4000	3.6	5/5	100	95	6.64

Corrected formula: For the 0% dead: 100(0.25/n) = 100(0.25/5) = 5

For the 100% dead: 100[(n-0.25)/n] = 100[(5-0.25)/5] = 95, n is the number of animals in the group

Table 3: Direct hemolysis of *Daboia russelli* venom can able to lyse the RBC's and the percentage of hemolysis was found to be 89.55%. *Rauvolfia serpentina* extract can able to reduce the lysis of RBC's and the percentage of hemolysis was found to be 34.32%. RBC + Distilled water served as 100% Hemolysis and RBC + PBS served as control.

Sample	OD of hemolysis	OD of Control (RBC + PBS)	OD of RBC + D.Water (100% Hemolysis)	% of Hemolysis
*Daboia russelli*venom	0.64	0.04	0.67	89.55
*Daboia russelli*venom + *Rauvolfia serpentina*	0.27	0.04	0.67	34.32

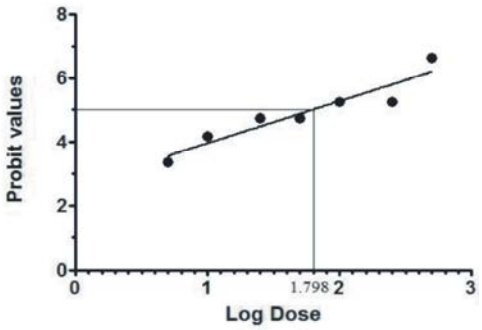

Fig 1: Calculation of Lethal dose (LD_{50}) of *Daboia russelli* venom in mice receiving various doses of *Daboia russelli* venom by Miller and Tainter method (n=5). The LD_{50} of *Daboia russelli* venom was found to be 0.628 µg/g.

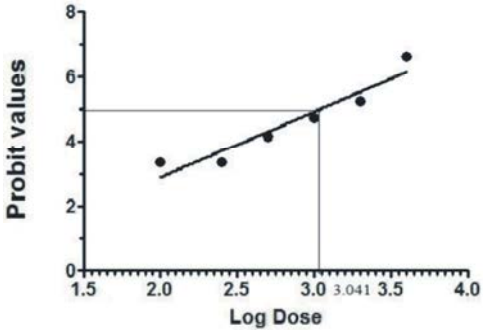

Fig. 2: Neutralization of Lethality (ED_{50}) by *Rauvolfia serpentina* against $3LD_{50}$ of *Daboia russelli* venom by Miller and Tainter method (n=5). The ED_{50} of *Rauvolfia serpentina* against $3LD_{50}$ of *Daboia russelli* venom was found to be 10.99 mg.

DISCUSSION

Snake bite is a major public health issue with high mortality rate worldwide. In India about 35,000 to 50,000 deaths occur every year due to snake bites. Many deaths occur especially in the rural areas of India, where the basic health facilities are poor and many victims fail to reach hospital in time, some even die before reaching hospital. The in-hospital mortality varies from 5 to 10% and the causes are acute renal failure, respiratory failure, sepsis, bleeding and others. Snake antivenom is a serum currently served as a antidote for snake bite envenomations. Due to

the cost and variety of side effects produced by antivenom serum it is important to find alternative source to treat snake bite envenomations. Although, use of plants against the effects of snakes bite has been long recognized, more scientific attention has been given since last 20 years. In our present investigation we check the snake venom neutralizing potential of *Rauvolfia serpentina* plant extract against *Daboia russelli* venom by *in vivo* and *in vitro* methods. Direct hemolysis of *Daboia russelli* venom produced 89.55% hemolysis and *Rauvolfia serpentina* extract can able to neutralize the hemolysis of RBC's produced by *Daboia russelli* venom upto 35%. In phospholipase activity (PLA_2) 8 µg of *Daboia russelli* venom was able to produce 11mm diameter hemolytic halo, which is considered to be 1Unit. *Rauvolfia serpentina* extract can capable of inhibiting PLA2 dependent hemolysis of sheep RBC's induced by *Daboia russelli* venom in a dose dependent manner. The medicinal plants *Thea sinensis Linn* and *Cordia verbenacea* effectively neutralized the phospholipase A2 activity induced by snake venoms [12]. In procoagulant activity the absence of clot formation showed the neutralizing ability of *Rauvolfia serpentina* extract. *In vivo* assessment of venom lethality (LD_{50}) of *Daboia russelli venom* was assessed and calculated by Miller and Tainter method and was found to be 0.628 µg/g. Venom-neutralizing potency test (ED_{50}) using *Rauvolfia serpentina extract* was assessed and was found to be 10.99 mg /3LD_{50} of venom. The tests for determining venom lethality (LD_{50}) and antivenom neutralizing capacity (ED_{50}) are currently the only validated means of assessing venom toxicity and antivenom neutralizing potency by both manufacturers and regulatory authorities worldwide. Due to excess of animal sacrifice alternative methods which minimum number of animals is required. FRAME (Fund for the Replacement of Animals in Medical Experiment) believes that the lethal dose test is unnecessarily cruel and scientifically invalid. Three alternative methods and these are: Fixed Dose Procedure (FDP), Acute Toxic Class method (ATC, Up-and-Down Procedure (UDP) [13, 14]. The acute oral toxicity of plant extract also observed and it was found that there was no any toxic effect on any mice due to the intake of crude plant extract. The result from this preliminary study indicates that *Rauvolfia serpentina* plant extract possess some compounds which can neutralizes the toxins present in *Daboia russelli* venom. Further investigations are needed for identification and purification of the active components involved in the neutralization of the snake venom.

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