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Indian Red Scorpion (*Mesobuthus tumulus) Venom* Neutralizing Potential of Aqueous Extracts of *Rauvolfia serpentina* and *Clitoria ternatea* by *in vitro* and *in vivo* Assays

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Abstract: Aqueous extracts of *Rauvolfia serpentina and Clitoria ternatea* were checked for their antidote properties against Indian Red Scorpion (*Mesobuthus tumulus)* venom by *in vitro* and *in vivo* methods. Direct hemolysis test was performed as *in vitro* screening assay in which *Rauvolfia serpentina and Clitoria ternatea* can able to neutralize the hemolysis of RBC's produced by *Mesobuthus tumulus* venom (87.35%) up to 30%. The Median Lethal Dose (LD_{50}) of *Mesobuthus tumulus* venom was found to be 4.365 µg/g. About 6.87mg of *Rauvolfia serpentina* and 7.78mg of *Clitoria ternatea* plant extracts were able to neutralize $3LD_{50}$ of *Mesobuthus tumulus* 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 all the selected plant extracts were >2000 mg/kg. These findings confirmed that *Rauvolfia serpentina and Clitoria ternatea* plant extracts contains some antitoxin compounds which inhibit the toxins present in *Mesobuthus tumulus* venom.

Key words: *Mesobuthus tumulus Venom* · Plant Extract · Lethality

INTRODUCTION

In India 86 species of scorpion have been identified out of which *Mesobuthus tamulus* and *Palamneus swammer-dami* are medically important. Except Hemiscorpius species, all lethal scorpions belong to the family Buthidae. In general for every case of snakebite, there may be 10 or more numbers of scorpion stings. There is no reliable statistics on the scorpion sting in India. According to the report of Tamil Nadu Health Systems Project, Govt of Tamil Nadu, Chennai, the scorpion stings are reported more from rural areas and the rural to urban ratio is approximately 3:1. Mostly stings occur between 6 P.M. to mid-night and between 6 A.M. to 12 Noon, which correlate very well with human activity [1]. The scorpion stings cases exceeds 1.23 million per year of which mortality cases was over 32250 [2]. Prazosin is a simple scientific pharmacological and physiological antidote to scorpion venom actions, it is easily available in rural. Administration of scorpion antivenom to patients presenting with cardiovascular manifestations of envenomation has not been conclusively shown to be of benefit [3]. Anti scorpion venom (ASV) also act as a promising antidote for serious cases of scorpion envenomation, but due to allergic reactions and various side effects present in antivenom serum therapy we are in need of finding alternative therapy that will neutralize scorpion envenomation. Many Indian medicinal plants are used as folk medicine for treatment of various diseases and poisonous bites generates considerable health and economic benefits. Various researches are worked on medicinal plants (Natural remedy) for treatment of snake and scorpion envenomations [4, 5]. Hence the present investigation explored the neutralizing potential of aqueous extracts of *Rauvolfia serpentina* and *Clitoria ternatea* against *Mesobuthus tumulus* venom by *in vitro* and *in vivo* methods.

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MATERIALS AND METHODS

Venom and Experimental Animals: The freeze-dried Indian Red Scorpion (*Mesobuthus tumulus)* venom 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 Plants and Preparation of Extracts: *Rauvolfia serpentina and Clitoria ternatea* plants were obtained from Arya Vaidya Pharmacy, Coimbatore and the extracts were prepared by the method of Uhegbu *et al*. [6] using distilled water as the solvent. The solution was filtered using filter paper (Whatman No. 1) and the extracts were lyophilised. The plant extracts were expressed in terms of dry weight.

Acute Oral Toxicity: Acute oral toxicity of *Rauvolfia serpentina* and *Clitoria ternatea* plant extracts were performed as per OECD guidelines 423. A limit test at 2000 mg/kg body weight of the extracts 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 vitro* Assessment of Venom Toxicity and Neutralization Assay**

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 10 minutes 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 20minutes. 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

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

***In vivo* Assessment of Venom Toxicity (LD_{50}):** The *in vivo* assessment of venom toxicity was done by LD_{50} range finding test and Median Lethal Dose (LD_{50}) assay. Various concentrations of *Mesobuthus tumulus* venom were prepared using saline and aliquots of a precise volume (0.2–0.5 ml) of each concentrations were injected, using one mouse per dose, by tail vein administration and deaths were recorded at 24 hours. This test is performed to narrow the range of venom doses required to formally estimate the toxic activity of the venom. In 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 by Miller and Tainter[7]. One venom LD_{50} is defined as the minimal amount of venom causing death in 50% of the mice [8].

Neutralization of Lethality (ED_{50})

ED_{50} Range-Finding Test: The Neutralization of Lethality was done by ED_{50} range finding test and the Median Effective Dose (ED_{50}) assay. Various dilutions of the plant extracts were made. The multiple of the *Mesobuthus tumulus* venom LD_{50} ($3LD_{50}$) was mixed with different doses of antivenom 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. In median effective dose (ED_{50}) assay various volumes of the antivenom were mixed with fixed amount of venom ("challenge dose", usually corresponding to $3LD_{50}$) and 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 6 mice 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 by Miller and Tainter [7]. The median effective dose (ED_{50}) of an antivenom is defined as the volume of antivenom that protects 50% of the mice injected [8].

RESULTS

In vitro and *in vivo* Antivenom potential of *Rauvolfia serpentina* and *Clitoria ternatea* extract against *Mesobuthus tumulus* were studied. Direct hemolysis of *Mesobuthus tumulus* venom produced 87.35% hemolysis and *Rauvolfia serpentina and Clitoria ternatea* can able to neutralize the hemolysis of RBC's produced by venom up to 30%. *In vivo* assessment of venom toxicity (LD_{50}) of *Mesobuthus tumulus venom* was assessed by LD_{50} range-finding test and median lethal dose (LD_{50}) assay using mice (18–20 g). LD_{50} of *Mesobuthus tumulus* was calculated by Miller and Tainter method and was found to be 4.365 µg/g (Table 1 and Fig. 1). Venom-neutralizing potency test (ED_{50}) using *Rauvolfia serpentina* and *Clitoria ternatea* extracts were assessed by ED_{50} range-finding test and median effective dose (ED_{50}) assay. The neutralization of lethality was done by preincubating constant amount of venom ($3LD_{50}$) with various dilutions of *Rauvolfia serpentina* and *Clitoria ternatea* extracts prior to injection. Calculation of ED_{50} of *Rauvolfia serpentina* and *Clitoria ternatea* against $3LD_{50}$ of venom was carried out by Miller and Tainter method. About 6.87mg of *Rauvolfia serpentina* and 7.78mg of *Clitoria ternatea* plant extracts were able to neutralize $3LD_{50}$ of *Mesobuthus tumulus* venom (Tables 2,3 and Figs. 2,3). 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 all the selected plant extracts were >2000 mg/kg.

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

Dose (µg/g)	Adjusted (Dose×100)	Log dose	Death/Total	Dead %	Corrected formula %	Probit
0.5	50	1.70	0/5	0	5	3.36
1	100	2.00	0/5	0	5	3.36
2.5	250	2.4	1/5	20	20	4.16
5	500	2.7	3/5	60	60	5.25
10	1000	3.0	4/5	80	80	5.84
25	2500	3.4	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 *Mesobuthus tumulus* venom by Miller and Tainter method (n=5).

Dose (µg/g)	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	1/5	20	20	4.16
5	500	2.7	2/5	40	40	4.75
10	1000	3	3/5	60	60	5.25
20	2000	3.3	4/5	80	80	5.84
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: Death Percentage of mice receiving various doses of *Clitoria ternatea* against $3LD_{50}$ of *Mesobuthus tumulus* venom by Miller and Tainter method (n=5).

Dose (µg/g)	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	1/5	20	20	4.16
5	500	2.7	2/5	40	40	4.75
10	1000	3	2/5	60	60	5.25
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

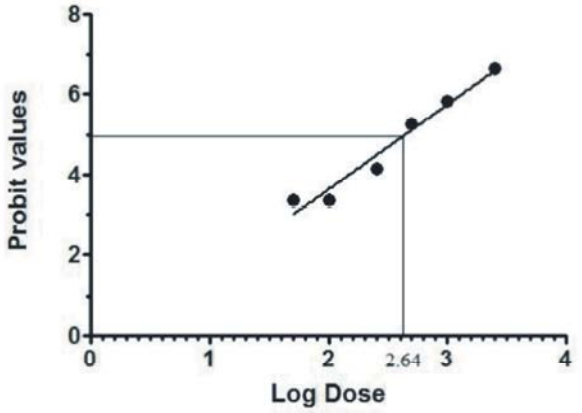

Fig. 1: Calculation of Lethal dose (LD_{50}) of *Mesobuthus tumulus* venom in mice receiving various doses of *Mesobuthus tumulus* venom by Miller and Tainter method (n=5). The LD_{50} of *Mesobuthus tumulus* venom was found to be 4.365 µg/g.

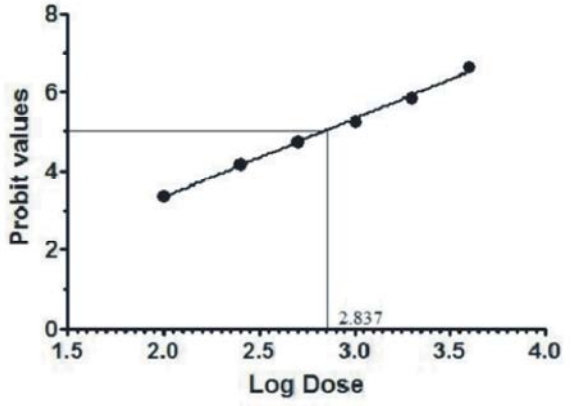

Fig. 2: Neutralization of Lethality (ED_{50}) by *Rauvolfia serpentina* against $3LD_{50}$ of *Mesobuthus tumulus* venom by Miller and Tainter method (n=5). The ED_{50} of *Rauvolfia serpentina* against $3LD_{50}$ of *Mesobuthus tumulus* venom was found to be 6.87mg.

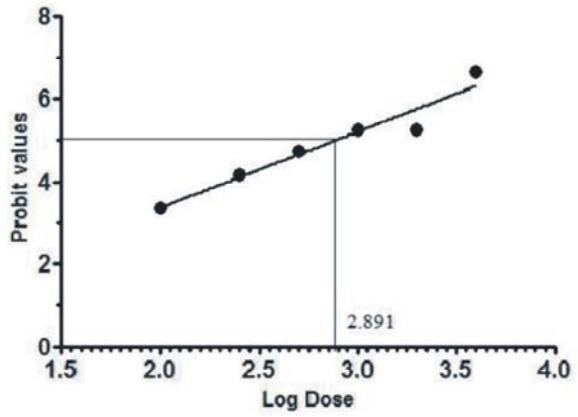

Fig. 3: Neutralization of Lethality (ED_{50}) by *Clitoria ternatea* against $3LD_{50}$ of *Mesobuthus tumulus* venom by Miller and Tainter method (n=5). The ED_{50} of *Clitoria ternatea* against $3LD_{50}$ of *Mesobuthus tumulus* venom was found to be 7.78 mg.

DISCUSSION

Scorpion sting is a life threatening medical emergency. According to the report from the Institute of child health, Madras Medical College, Chennai, there are nearly 1900 cases were recorded between 1980 and 1999 and the fatality rate varied from 4 to 7%. There are 727 cases of scorpion stings treated during the period of 2000-2007 which included 406 males and 321 females [M: F= 4:3]; the casualty among them were 11 and 8 respectively. Dr.H.S. Bawaskar, a private practitioner from Maharashtra has introduced the usefulness of alpha blocker in scorpion sting nearly 25 years ago. This has been accepted globally now in the treatment of scorpion sting [1]. Many Indian medicinal plants are recommended for the treatment of snake and scorpion envenomations. In our present investigation inhibitory properties of

Rauvolfia serpentina and *Clitoria ternatea* extract against *Mesobuthus tumulus* were studied. *In vivo* assessment of venom toxicity and neutralization by plant extracts were done by Direct hemolysis method and the results showed that *Mesobuthus tumulus* venom produced 87.35% hemolysis and *Rauvolfia serpentina* and *Clitoria ternatea* can able to neutralize the hemolysis of RBC's produced by venom up to 30%. 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 [9]. Based on this the *in vivo* assessment of venom toxicity (LD_{50}) of *Mesobuthus tumulus venom* was done by LD_{50} range finding test and Median Lethal Dose (LD_{50}) assay. The Median Lethal Dose (LD_{50}) of *Mesobuthus tumulus* was found to be 4.365 µg/g. Venom-neutralizing potential of *Rauvolfia serpentina* and *Clitoria ternatea* extracts were assessed by the median effective dose (ED_{50}) assay. About 6.87mg of *Rauvolfia serpentina* and 7.78mg of *Clitoria ternatea* plant extracts were able to neutralize $3LD_{50}$ of *Mesobuthus tumulus* venom. In Acute Oral Toxicity all animals survived and appeared active and healthy throughout the study. Similar study was carried out by Brahmane *et al*. [10] using *Andrographis paniculata* and reported that the acute toxicity and *in vivo* neutralization study, plant extract at the dose of 1 g/kg and 2 g/kg resulted in a mean survival of 62.667 min and 39.333 min respectively. These findings confirmed that *Rauvolfia serpentina and Clitoria ternatea* plant extracts possess some antidote compounds which inhibit the toxins present in *Mesobuthus tumulus* venom. Further studies are essential to identify the active compound which can able to neutralize all the lethal activity of *Mesobuthus tumulus* venom.

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