



Research Article

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IN VIVO AND IN VITRO NEUTRALIZING POTENTIAL OF STEREOSPERMUM TETRAGONUM PLANT EXTRACT AGAINST RUSSELL'S VIPER (*DABOIA RUSSELLI*)

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Received on: 24/02/17 Accepted on: 23/04/17

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DOI: 10.7897/2277-4343.082125

ABSTRACT

Snake bite needs medical emergencies and administration of specific anti venom. As anti venom induces adverse reactions in human beings, snake venom neutralizing potential of *Stereospermum tetragonum* plant extract was tested in the present research by *in vitro* and *in vivo* methods against Russell's viper (*Daboia russelli*) venom. *In vitro* assessment of venom toxicity and neutralization assays was carried out. *In vitro* assessment of venom toxicity and neutralization assays was carried out. The plant extracts of *Stereospermum tetragonum* were used to evaluate the *in vitro* neutralization tests like direct hemolysis assay, phospholipase activity and Pro coagulant activity. The *in vivo* assessment of venom lethality (LD₅₀) of Russell's viper (*Daboia russelli*) venom was found to be 0.602µg. *Stereospermum tetragonum* plant extract was effectively neutralized the venom lethality and effective dose (ED₅₀) was found to be 10.47mg/3LD₅₀ of russell's viper (*Daboia russelli*) venom. *Stereospermum tetragonum* plant extract was found effective in neutralizing all the toxic effects induced by the venom. The study concludes that further investigations are needed for identification and purification of the active components involved in the neutralization of the snake venom.

Keywords: *Stereospermum tetragonum*, Russell's viper (*Daboia russelli*), phospholipase activity, procoagulant.

INTRODUCTION

Snakebites represent a public health hazard that leads to high morbidity and mortality in the Indian subcontinent¹. More than 2000 species of snake in this world and 216 species in India have been identified among which 52 are highly venomous. Cobra (*Naja naja*), Krait (*Bangarus caeruleus*), Russell's viper (*Daboia russelli*) and Saws scaled viper (*Echis carinatus*) are commonly considered be poisonous snakes in the Indian ethnicity². Elapidae and Viperidae are two families of species which cause human mortality world wide³. The Elapidae family contains the cobras and their relative species. All types of vipers come under the family Viperidae. *Daboia russelli* (Indian sub species of *Russell's viper*) appears to be the commonest cause of fatal snake bite in southern India⁴. Envenomation of snake bite is caused due to the nature of enzymatic and non-enzymatic toxic compounds present in the poison glands⁵. Enzymes present in snake venom hydrolyze protein and membrane components which lead to tissue necrosis and blood clotting⁶. Phosphodiesterase A₂ causes hemolysis by a lysine cell membrane of RBCs. Oxidases and proteases are used for digestion. Snake venom is one of the most amazing and unique adaptations of snakes on animal planet. Venoms are mainly toxic modified saliva consisting of a complex mixture of chemicals called enzymes found in snake poison⁷. Antivenom are usually hyper immunes era collected from animals which bind and inactivate venom components. Till date, antibodies produced in horses are the only source of ant venom, a process of time consuming. Moreover, antibodies need to store in low temperature conditions that lack in rural areas of developing countries⁸. Monovalent antiserum is not available and the health center is usually far and few in number. Snake venom antiserum

has administration problems too, as the exact dosage is also not clear to overcome these drawbacks, there is a great need to search, to develop new affordable and suitable antidote against snake bite⁹. Alkaloids, acids, coumestins, steroids, glycoprotein's, glycosides, phenols, tannins, terpenoids, quinonoid xanthine and miscellaneous chemical groups present in the plant extracts possess an effective antidote against snake venom envenomation¹⁰. Though the defining mechanism of herbal based antidote therapy is not yet delineated, the majority of the herbal compounds tend to neutralize the toxic venom constituents¹¹. Neutralization of venom by phytoconstituents includes the process of enzyme inactivation, chelation, adjuvant actions, anti-oxidation and protein folding^{12, 13}. The plant constituents were identified to play a major role for neutralizing the effects of some types of snake venoms. The snake bite management was accomplished using herbal plants either using a single herbal ingredient or by using a combination of herbal plants. According to Kuntal Das (2009)¹⁴, the recent scientific investigations have confirmed the efficacy of many herbal preparations against snake bites. The most effective herbs were also reported as relatively non-toxic and have substantial documented efficacy. Among them some herbs are *Aristolochia* species, *Cissus assamica*, *Echinacea* species, *Guiera senegalensis*¹⁵, *Hemidesmus indicus*, *Parkia biglobosa*¹⁶, *Securidaca longipedunculata*, *Thea sinensis*, *Tamarindus indica*, *Trianosperma tayuya*, *Withania somnifera*¹⁷. It was reported that each plant and its specific parts like root, leaves, barks etc. has herbal antidote properties against specific snake venoms. It was investigated through various instrumental analyses like GC-MS and LC-MS, HPTLC, etc. Specific plant constituents or its phytochemicals play a major role in providing the antidote properties rather than the whole plant itself¹⁸.

Herbal compounds active against snake envenomation were already reported. Herbal plants like *Hemidesmus indicus*, *Pimpinella anisum*, *Salix alba* contains aristolochic acid, anisic acid, salicylic acid, respectively¹⁹. Alkaloids like atropine and APLAI as herbal antidote is present in *Dendroaspis polylepis* and *Azadirachta indica*²⁰. Ethanol, a pterocarpanes is present in *Harpalyce brasiliensis*²¹. Terpenoids like glycyrrhizin, neoclerodane, ursolic acid is present in *Glycyrrhiza glabra*, *Baccharis trimera* and *Eriobotrya japonica*²². In the present research, *Stereospermum tetragonum* significant medicinal herb was screened for its antivenom properties. *Stereospermum tetragonum* is a large straight stemmed deciduous tree 18-30 m height and 2.8 m girth found throughout in moist regions of India up to an altitude of about 1200m, chiefly in deciduous forests²³. The plants are having bitter, astringent and acrid property. The medical significance of *Stereospermum tetragonum* was earlier reported. The plants are used as anodyne, appetizer, constipating, diuretic, litho tropic, expectorant, cardio tonic, aphrodisiac, anti-inflammatory, antibacterial, febrifuge tonic, antiemetic, antipyretic. The decoction of plant is used in the treatment of asthma and cough²⁴. The gas chromatography of the plants revealed other significant constituents like Carbonochloridic acid, methyl (2-propynyl) hydrazine acetic acid, phytosterol and 5-n-pentadecyl-2,4-dinitro-1-hydroxy-benzene. Although the plant has been a widely used as folklore medicine with reportedly high diuretic and anti-inflammatory properties, the anti venom potential remains still uncharacterized. Hence, in this research, the neutralization efficacy of *Stereospermum tetragonum* plant extract against Russell's viper (*Daboia russelli*) snake venom was determined by both *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Snake venom

The freeze-dried snake venom powders of *Daboia russelli* were obtained from Irula's Snake Catchers Industrial Co-operative Society Limited Chennai, Tamil Nadu, India. The collected powders were stored at 4°C. Stock solution was prepared by dissolving 1mg of lyophilized venom in 1ml of physiological saline (1mg/ml).

Collection and authentication of plant material

Medicinal plant *Stereospermum tetragonum* was collected from Arya Vaidya Pharmacy, Coimbatore district, Tamil Nadu, India. Aqueous extracts were prepared by the method of Uhegbu using distilled water. *Stereospermum tetragonum* (BSI/SRC/5/23/2015/Tech./164) authenticated by Dr.K.Althaf Ahamed Kabeer, Scientist 'D' in charge, Botanical Survey of India Southern Regional Centre, Coimbatore.

Preparation of Extracts

About 20g of powdered sample of the herb was extracted by soaking in 180ml of distilled water in a beaker, stirred for about 6min and left over night. 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.

Acute Oral Toxicity

Acute oral toxicity of all the selected plant extracts was performed as per OECD guidelines 423. A limit test at 2000mg/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 gavage. 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.

(Ethics committee approval number: JSSCP/IAEC/CADRAT/07/2013)

In vitro Assessment of Venom Toxicity and Neutralization assays

Direct Hemolysis Assay

The hemolytic acts of *Daboia russelli* venom and plant extracts were studied *in vitro* by using RBC. Briefly, 5ml of citrated blood was centrifuged for 10min at 900rpm. The supernatant was poured off and the pellet was washed twice with physiological salt solution. 5ml of physiological saline and 0.5ml of RBC mixture served as a control. 5ml of distilled water with 0.5ml of washed RBC was used for 100% hemolysis. 5ml of venom/extract and 0.5ml of washed RBC served as experimental sample. The tubes were put in a thermostat for 1hr at 37°C and centrifuged at 2000rpm for 20m. The supernatant fluid was poured off to separate tubes to measure the optical density using spectrophotometer at a wavelength of 540nm against water.

Indirect Hemolysis Assay (PLA₂ activity)

Phospholipase A₂ activity was measured using an indirect hemolytic assay on agarose-erythrocyte-egg yolk gel plate by the method²⁶. Increasing concentrations of *Daboia russelli* venoms (in µg) were 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 concentration of venom, which produced hemolytic halo of 11mm diameter. The efficacy of *Stereospermum tetragonum* extracts in neutralizing the phospholipase activity was estimated by mixing a constant amount of venom (µg) with different amount of plant extracts (µl) and incubated for 30 min at 37°C. Then, aliquots of 10 µl off to the mixtures were added to the 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

The procoagulant activity was done according to the method described by Laing *et al.*, (1992)²⁷. Various amounts of venom dissolved in 100µl PBS (pH 7.2) were 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 30min at 37°C. Then 0.1ml of mixture was added to 0.3ml of citrated plasma and the clotting times were recorded. In control tubes, plasma was incubated with either venom alone or plant extract alone. Neutralization was expressed as effective dose (ED₅₀), defined as the ratio µl anti venom (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.

Proteolytic activity

Proteolytic activity was determined according to the method²⁸ using 2% casein as substrate in 0.02M Tris-HCl buffer (pH 8.5). Venom 200µg (1mg/ml) and different dilutions of plant extract 200µg, 250µg, 300µg were pre-incubated with 1ml of substrate for 2h at 37 °C. The undigested casein was precipitated by the addition of 1.5ml of 0.44M trichloroacetic acid (TCA) and centrifuged. The digested casein in the supernatant was determined using Folin ciocalteu's reagent. Venom without plant extract was considered as control or 100% activity.

In vivo assessment of venom toxicity and anti-venom effect of plant extracts lethal toxicity

Various doses of venom in 0.2ml of physiological saline were injected into the tail vein of mice, using groups of 3-5 mice for 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 plant extracts were determined against 2LD₅₀ of *Daboia russelli* venom. Various amounts of plant extracts (µl) were mixed with 2LD₅₀ of venom sample and incubated at 37°C for 30min and then injected intravenously into mice. 3-5 mice were used in each anti venom dose. Control mice received the same amount of venom without anti venom (plant extracts). The median Effective Dose (ED₅₀) calculated from the number of deaths within 24 hours of injection of the venom/anti venom mixture. ED₅₀ was expressed as µl anti venom/mouse and calculated by probit analysis.

(Ethics committee approval number: JSSCP/IAEC/PH.COLOGY/03/2013-2014)

Table 1:-Calculation of LD₅₀ of *Daboia russelli* venom in 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
0.025	2.5	0.4	0/5	0	5	3.36
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:Calculation of ED₅₀of *Stereospermum tetragonum* against *Daboia russelli* venom in mice 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	200	2.3	0/5	0	5	3.36
4	400	2.6	1/5	20	20	4.16
8	800	2.9	2/5	40	40	4.75
16	1600	3.2	3/5	60	60	5.25
25	2500	3.4	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

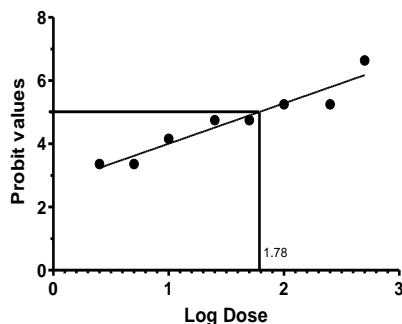


Figure 1: Calculation of Lethal dose(LD₅₀) of *Daboia russelli* venom by Miller and Tainter method and LD₅₀ of *Daboia russelli* is found to be = 0.602 µg/g

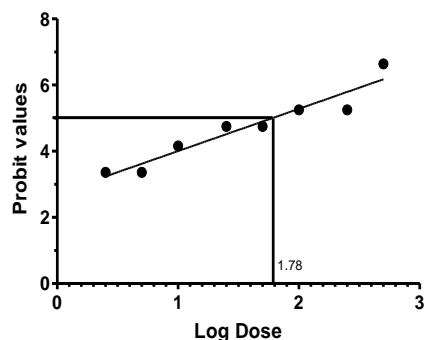


Figure 2: Effective dose of *Stereospermum tetragonum* against 3 LD₅₀ of *Daboia russelli* by Miller and Tainter(n=5)

RESULTS AND DISCUSSION

Snakebite is always considered to be as a major health hazard which leads to the high mortality rate world-wide. The true global incidence of snake bite and associated mortality is difficult to estimate. Anti-snake venom remains the specific antidote for snake venom poisoning with different limitations in its usage. It consumes lot of time for the development and expensive. Since it contains horse immunoglobulin's, it leads to cause complement mediated side effects, like serum sickness and anaphylactic shock. Due to these limitations of anti-venoms, since last 20 years more scientific attention for using plants and medically significant herbs against different snake bites was given importance²⁹. More interestingly, many Indian medicinal plants were recommended for the treatment of snakebites world-wide³⁰. The Anti venom potential of *Stereospermum tetragonum* plant extract against *Daboia russelli* venom was investigated in the present study by both *in vitro* and *in vivo* experiments. Similarly, Soares *et al.*, (2005)³¹ reported that bioactive compounds in the plant extracts will bind to divalent metal ions, required for enzymatic activities. Thus, weaken the protease-metal ion interaction resulting in inhibition of the proteolytic activity.

In vitro Assessment of Venom Toxicity and Neutralization assays

The inhibitory effect of *Stereospermum tetragonum* extracts on the acetyl cholinesterase activity of venom was determined *in vitro*. During this experiment, direct hemolysis of *Daboia russelli* venom produced 93.50% hemolysis. *Stereospermum tetragonum* plant extract neutralized the hemolysis of RBC's produced by the venom up to 24%. In phospholipase activity (PLA₂) 10µg of *Daboia russelli* venom was able to produce 11mm diameter hemolytic halo, which is considered to be 1Unit. *Stereospermum tetragonum* extract can capable of inhibiting PLA₂ dependent hemolysis of sheep RBC's induced by *Daboia russelli* venom in a dose dependent manner. In procoagulant activity 100µg of *Daboia russelli* venom was found to clot human citrated plasma in the 60s. In the neutralization assay, the absence of clot formation shows the neutralizing ability of plant extract. High concentration of venom caused rapid clotting that required very high concentration of plant extracts to neutralize.

In vivo assessment of venom toxicity and anti-venom effect of plant extracts lethal toxicity

In vivo assessment of venom lethality (LD₅₀) of *Daboia russelli* venom was assessed and calculated by Miller and Tainter

method. The tests for determining venom lethality (LD₅₀) and anti venom neutralizing capacity (ED₅₀) are currently the only validated means of assessing venom toxicity and anti venom neutralizing potency by both manufacturers and regulatory authorities worldwide. *In vivo* assessment of venom toxicity (LD₅₀) of the venom was assessed by LD₅₀ range-finding test and the median lethal dose (LD₅₀) assay using mice (18-20g). LD₅₀ of *Daboia russelli* venom was calculated and found to be 0.602µg/g. (Table 1, Figure 1) Venom-neutralizing potency test (ED₅₀) using *Stereospermum tetragonum* extract was carried out by pre-incubating constant amount of venom (2LD₅₀) with various dilutions of the plant extracts prior to injection. Calculation of ED₅₀ of *Stereospermum tetragonum* against 3LD₅₀ of venom was done by Miller and Tainter method and found to be 10.47mg/3LD₅₀ venom. (Table: 2, 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 LD₅₀ of *Stereospermum tetragonum* plant extract was >2000mg/kg. 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 *Stereospermum tetragonum* plant extract possesses some compounds which can neutralizes the toxins present in *Daboia russelli* venom.

CONCLUSION

The *in vitro* enzymatic analysis reveals that the *Stereospermum tetragonum* plant extract could inhibit most of the toxic enzymes of the *Daboia russelli*. The result from *in vivo* and *in vitro* analysis showed that *Stereospermum tetragonum* plant extract possesses neutralizing potential against venoms, in present investigation *Daboia russelli* neutralize by the aqueous extract of *Stereospermum tetragonum* shows a good anti-venom activity. In a further study to conclude the mechanism of action and to find out the active compounds responsible for its action.

ACKNOWLEDGEMENT

I would like to thank my guide Dr. S.Meenachisundharam and the management of NASC, Coimbatore for providing the premises for carrying out this research work and Dr. M. Palinisamy, Scientist 'D' in charge, Botanical Survey of India Southern Regional Centre, Coimbatore for identification of plant.

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Cite this article as:

Sreedevi N.S et al. *In vivo* and *In vitro* neutralizing potential of *Stereospermum tetragonum* plant extract against Russell's viper (*Daboia russelli*). Int. J. Res. Ayurveda Pharm. 2017;8(Suppl 2):266-270 10.7897/2277-4343.082125

Source of support: Nil, Conflict of interest: None Declared

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