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Article

Neutralizing potential of *Rauvolfia serpentina* root extract against *Naja naja* venom

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Snake bites are a serious health hazard occurs throughout the world especially in tropical countries like India. Anti-Snake Venom Serum is the only remedy available to treat snake bite victims successfully till date. Infusion of ASV may lead to adverse reactions ranging from severe itching of the skin, hives to potentially serious allergic reactions. Considering all above difficulties research workers all over the world is constantly in search of a cheap and readily available easy formulate remedy for treating snake bite victims. In present study aqueous extract of *Rauvolfia serpentina* root was checked for the antidote properties against *Naja naja* venom by *in vitro* and *in vivo* methods. Various *in vitro* neutralization tests like Acetyl cholinesterase, Protease and ATPase activity of *Naja naja* venom were carried out and the root extract was neutralized all the toxic effects induced by the venom. The *in vivo* assessment of venom lethality (LD_{50}) of *Naja naja* venom was found to be 0.301 µg. The aqueous root extract was effectively neutralized the venom lethality and effective dose (ED_{50}) was found to be 12.88 mg/ $3LD_{50}$of *Naja naja* venom. LC-MS analysis from root extract of *Rauvolfia serpentina* was done for confirmation of the bioactive compounds.

Keywords: Snake bite. *Naja naja. Rauvolfia serpentina.* Antivenom compounds.

INTRODUCTION

Snakebite envenoming is a neglected tropical disease which requires immediate consideration. Every year 2.5 million people are bitten by snakes with 85,000 deaths (Gutierrez *et al.*, 2010). Agriculturists and their families living in rural areas of the country are the most affected community, thus snakebite is represented as 'disease of poverty' (Harrison *et al*., 2009). A total number of snake species identified is about 2,000 to date and nearly 300 species of them are venomous snakes, which prevail over all parts of the world except Antarctica (Mohapatra *et al*., 2011). The four major species of venomous snakes ubiquitous in India known as "Big four" are considered responsible for life-threatening envenomation around the country (Mukherjee, 2012). The most common enzymes in snake venoms are phospholipase A_2s (PLA_2s), serine proteinases, metalloproteinase, acetyl cholinesterase (AChEs), l-amino acid oxidases, nucleotides (5′-nucleotidase, ATPase, phosphodiesterases and DNase) and hyaluronidase. Snake venoms are the most abundant source of all these enzymes (Kang *et al.,* 2011). The venom of *Naja naja* is neurotoxic, they affect the victim's central nervous system and cause heart failure. The venoms are rich in post synaptic neurotoxins called alpha-bungarotoxin and cobratoxin. Venom binds particularly to Acetylcholine receptors, prevents the interaction between Acetylcholine and receptors on post synaptic membrane result in neuromuscular blockade (Bawaskar, 2014). Antiserum is the only therapeutic agent available throughout the world. The antiserum development is a costly, time-consuming process requiring ideal storage conditions. Absolute specificity is an issue in management with antiserum. Snake venom antiserum does not provide adequate safety against venom induced local pain, local bleeding swelling and difficulty including wound necrosis, hemorrhage, necrosis and nephrotoxicity (Sarkhel *et al*., 2011). Over the years, many attempts have been made for the advance of snake venom antagonists

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particularly from plants sources since there are limitations on the expansion of anti-sera (Khan *et al*., 2014). In folk medicine, many botanical species are employed for the treatment of snakebites in communities that lack rapid access to serum therapy especially in developing countries. Herbal components play an important role in management and controlling of venomous snake bite (Vijaya *et al*., 2013).

Rauvolfia serpentina (L.) Benth. ex Kurz. (*Ophioxylon serpentinum* L.) is an evergreen, perennial shrub with maximum height up to 60 cm (Deshmukh, Dhanashree, Patil *et al*., 2012). The plant belongs to the family Apocynaceae and occurs to habitats of tropical and subtropical regions. The plant is commonly known as Sarpagandha, Chandrabagha, Snake root plant, Chotachand, Chandrika and Harkaya etc (Mallick, Jenna, Samal, 2012). The roots of *Rauvolfia serpentina* are used in Ayurvedic medicines as a treatment for curing hypertension, insomnia, mental anxiety, gastrointestinal disorders, anticipation epilepsy, trauma, anxiety, stimulation, schizophrenia, sedative insomnia and psychosis (Rathore *et al*., 2012). It is used as an antidote to snakes and bites of other toxic insects (Ghani, 1998). Rhizome and leaf decoction are orally given in snake bite in the rural areas of Kanyakumari district, India (Jeeva *et al*., 2006).

The present investigation explored *Naja naja* venom neutralizing activity of *Rauvolfia serpentina* root extracts by *in vitro*, *in vivo* methods and antivenom compounds were identified using LCMS analysis.

MATERIAL AND METHODS

Collection and authentication of plant material:

Rauvolfia serpentina (L.) Benth. ex Kurz. (*Ophioxylon serpentinum* L.) belongs to the family Apocynaceae was collected from Anakkal region, Malampuzha, Palakkad district, Kerala after questionnaire with tribal people and from vaidyas in and around Palakkad district. It was authenticated by Dr. Althaf Ahamed Kabeer. Scientist 'D'.Botanical Survey of India Southern Regional Centre. Coimbatore (Herbarium voucher specimen number 1160).

Preparation of extract

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 over night. Thereafter, the solution was filtered using filter paper (What man No. 1) and the extracts were evaporated to dryness under reduced pressure in 40 °C. The plant extracts were expressed in terms of dry weight.

Extraction yields (%) = (weight of freeze dried extract *100/weight of original sample).

Extraction yields of *Rauvolfia serpentina* is 2.5%

Snake venom

The freeze-dried snake venom powders of *Naja naja* were obtained from Irula's Snake Catchers Industrial Co-operative Society Limited Chennai and was stored at 4 °C. Stock solution was prepared by dissolving 1 mg of lyophilized venom in 1mL of physiological saline (1 mg/mL). (Ethics committee approval number: JSSCP/IAEC/PH.D/PH.COLOGY/02/2014-15)

Acute oral toxicity

Acute oral toxicity of all the selected plant extracts was performed as per OECD guidelines 423. A limit test of 2000 mg/kg body weight of the extracts was administered. Briefly, two thousand milligrams of the test substance per kilogram of body weight were 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 assays

Acetyl cholinesterase activity

Acetyl cholinesterase inhibition assay was carried out according to the modified method of Ellman *et al*. (1961). 200 µg of venom (1 mg/mL) was pre-incubated (1 h) with different concentrations of plant extract and supernatant was added to the assay mixture which consists of 100 µL of 75 mM acetylcholine idodate in 1 mL of phosphate buffer. The activity was measured by taking the absorbance at 412 nm. Venom without plant extracts was considered as control or 100% activity

Inhibition % = control-test/control x100

Proteolytic activity

Proteolytic activity was determined according to

the method Satake, Murata and Suzuki (1963). Using 2% casein as substrate in 0.02 M Tris-HCl buffer (pH 8.5). Venom 200 µg (1 mg/mL) and different dilutions of plant extract were pre- incubated with 1 mL of substrate for 2 h at 37 °C. The undigested casein was precipitated by the addition of 1.5 mL of 0.44 M trichloroacetic acid (TCA) and centrifuged. The digested casein in the supernatant was determined using Folin ciocalteu's reagent. Venom without plant extracts was considered as control or 100% activity.

Inhibition % = control-test/control x100

ATPase activity

ATPase activity was measured according to the modified method of Kini and Gowda (1982). *Naja naja* venom 200 µg (1 mg/mL) were pre-incubated with different concentrations of plant extract of *Rauvolfia serpentina* root for 30 minutes. To the reaction, 1 mL of assay mixture (750 µL of 0.1 M Tris pH 7.5, 100 µL of 0.1 MgCl2, 50 µL of 0.1 M ATP, and 100 µL of BSA) was added with gentle shaking at 37 °C and stopped at a certain time (1 h) by adding 1 mL of SDS solution. The inorganic phosphate formed was determined by phosphate determination method by taking 400 µL of sample along 600 µL of TCA and incubated separately for 10 min at 37 °C followed by centrifugation at 1500 rpm for 10 min. About 500 µL of supernatant was added together with 500 µL of ferrous sulphate-ammonium molybdate reagent and the absorbance was measured at 820 nm within 2 h for every 10 minutes of intervals. Reaction mixture without plant sample was referred as control or 100% activity. Inhibition reaction was calculated in terms of percentage (100%). Na,K-ATPase were mainly used.

Inhibition % = control-test/control x100

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

The median lethal dose (LD_{50}) of *Naja naja* venom was determined according to the method of Randhawa (1944).Various doses of venom in 0.2 mL of physiological saline were injected into the tail vein of mice, using groups of 3-5 mice for each venom dose. The LD_{50} was calculated with confidence limit at 50% probability by the analysis of deaths occurring within 24 h of venom injection.

The anti-lethal potentials for plant extract were determined against $2LD_{50}$ of *Naja naja* venom. Various amount of plant extracts (µL) were mixed with $2LD_{50}$ 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 (ED_{50}) calculated from the number of deaths within 24 h of injection of the venom/antivenom mixture. ED_{50} was expressed as µL antivenom/mouse and calculated by probit analysis (Miller, Tainter, 1944).

LC-MS analysis

Phytochemical screening for compounds present in the aqueous extract from *Rauvolfia serpentina* root was carried out using LC Column: Reverse Phase C-18. The chromatographic separation was performed on a Phenomenex RP C-18 (25 cm x2.5 mm) at Column temperature: 25 °C. A gradient Electronic Spray Ionization mode was performed at a flow rate of 2 mL/mins. The mobile phase: water: methanol (40:50). The volume of injection was 10 µL. Mass spectrometry data were obtained both positive and negative ionization modes. Class V P Integrated Soft Ware was used for the MS analysis and analyzed samples were compared with METWIN 2.0 library. These antivenom compounds were detected by PASS software. The computer program PASS (Prediction of Activity Spectra for Substances) was designed to predict many kinds of biological activity simultaneously based on the structural formulae of chemical compounds (Filimonov, Poroikov, Karaicheva, 1995; Poroikov, Filimonov, 1996).

Statistical analysis

Statistical evaluation was performed using XL stat 2008 and SPSS 10 Software<0.005 was considered statistically significant.

RESULTS

Inhibition of Acetyl cholinesterase activity

The aqueous extract of the plant was taken in different dilutions starting from 200 µg to 300 µg with triplicate experiments. Maximum of Acetyl cholinesterase inhibition (60.29%) was occurred at 300 µg concentration of venom and aqueous extract of plant respectively. The activity was calculated in terms of percentage of inhibition compared to venom pre-incubated with different amounts of plant extract and venom with substrate. The enzyme reaction was observed for every 10 minutes intervals at 412 nm. Acetyl cholinesterase activity of the venom was considered as 100%. (Figure 1) and (Table I).

TABLE I - Acetylcholinesterase activity on *Naja naja* venom

Venom sample	Concentration of plant extract(µg)	OD and Inhibition %	*Rauvolfia serpentina* root	Control
Naja naja	200	OD	0.53	
		Inhibition %	22.05	.68
	250	OD	0.37	
		Inhibition %	45.48	.68
	300	OD	0.27	
		Inhibition %	60.29	.68

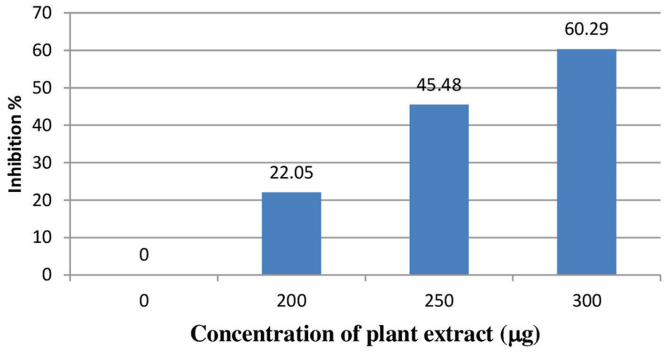


FIGURE 1 - Acetylcholinesterase inhibition assay.

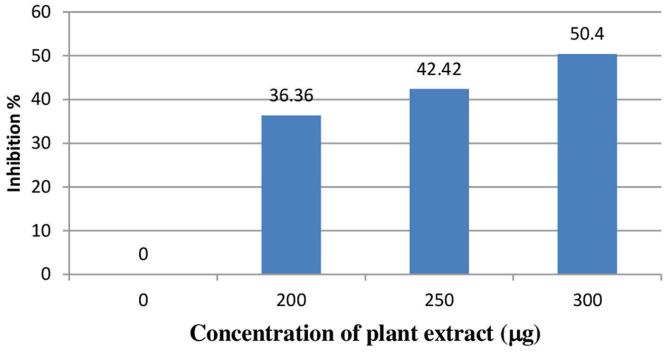


FIGURE 2 - Protease inhibition assay.

Inhibition of protease activity

To assess the in vitro antagonism of protease, the venom degrades the substrate (casein) into peptide precipitation could be observed at 600 nm. Maximum of protease inhibition (50.40%) was occurred at 300 µg concentrations of venom and aqueous extract of plant respectively. From the results it was observed that increased amount of plant extract could increase the inhibition of protease of cobra activity (Figure 2 and Table II).

Inhibition of ATPase activity

ATPase inhibition was calibrated by liberation of inorganic phosphate with of positive control of venom (200 µg) and substrate as ATP (10 µm). Different concentrations of venom and substrate were used for this reaction. The same concentration of venom 200 µg with different amounts of active aqueous extract from the plant (200 µg to 300 µg) was pre- incubated for the reaction. Maximum inhibition up to 58.76% has been noted at highest amount of plant concentration (Figure 3 and Table III).

In vivo methods

In vivo assessment of venom toxicity (LD_{50}) of *Naja naja* 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 *Naja naja* venom was calculated by Miller and Tainter method and was found to be 0.301 µg/g.

TABLE II - Protease activity on *Naja naja* venom

Venom sample	Concentration of plant extract(µg)	OD and Inhibition %	*Rauvolfia serpentina* root	Control
Naja naja	200	OD	0.42	
		Inhibition %	36.36	.65
	250	OD	0.38	
		Inhibition %	42.42	.65
	300	OD	0.33	
		Inhibition %	50.40	.65

TABLE III - Atpase activity on *Naja naja* venom

Venom sample	Concentration of plant extract(µg)	OD and Inhibition %	*Rauvolfia serpentina* root	control
Naja naja	200	OD	0.57	
		Inhibition %	41.23	.97
	250	OD	0.52	
		Inhibition %	46.39	.97
	300	OD	0.40	
		Inhibition %	58.76	.97

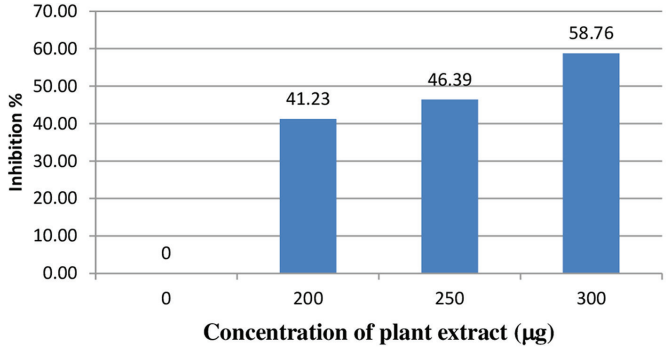


FIGURE 3 - ATPase inhibition assay.

(Table IV and Figure 4).Venom neutralizing potency tested (ED_{50}) using *Rauvolfia serpentina* root extract was carried out by pre incubating constant amount of venom ($3LD_{50}$) with various dilutions of *Rauvolfia serpentina* root extract prior to injection. Calculation of ED_{50} of *Rauvolfia serpentina* root of $3LD_{50}$ of venom was found to be 12.88 µg against *Naja naja* venom (Table V and Figure 5). All animals survived and appeared active and healthy throughout acute oral toxicity study. There were no signs of gross toxicity, antagonistic pharmacological effects or uncommon behavior. Based on the above findings, the LD_{50} of *Rauvolfia serpentina* root extract was > 2000 mg/kg.

LCMS analysis

Various compounds were identified by LCMS analysis which includes D-glucuronic acid, triacontanol, alppha-ionine, hydroquinone, reserpinine, reserpine, ascorbic acid, gallic acid, hydrangenol and oleic acid and their activities were determined using PASS software. Among the compounds, D-glucuronic acid, triacontanol, reserpine, gallic acid and oleic acid possess antivenom activity due to inhibition of various snake venom enzymes activities like phospholipase A_2 inhibition, ATPase inhibition, nucleotidase inhibition, 5'-nucleotidase inhibition, L-aminoacid oxidase inhibition, phosphodiesterase inhibition activity and have antidote activity (Table VI and Figure 6).

TABLE IV - Calculation of LD_{50} of *Naja naja* venom in mice receiving various doses of *Naja naja* venom by Miller and Tainter method (n=5)

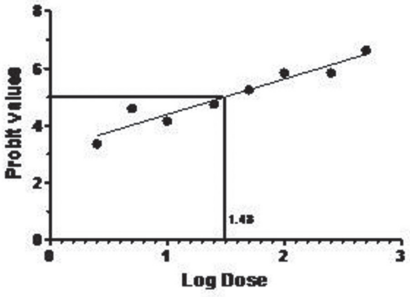
Dose (µg/g)	Adjusted (Dose×100)	Log dose	Death/Total	Dead %	Corrected formula %	Probit values
0.025	*2.5*	0.4	0/5	0	5	3.36
0.05	*5*	0.7	1/5	0	20	4.16
0.1	*10*	1	1/5	20	20	4.16
0.25	25	1.4	2/5	40	40	4.75
0.5	50	1.7	3/5	60	60	5.25
1.0	100	2.0	4/5	80	80	5.84
2.5	250	2.4	4/5	80	80	5.84
5.0	500	2.7	5/5	100	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 V - Calculation of ED_{50} of *Rauvolfia serpentina* against *Naja naja* 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
2.5	*250*	2.4	0/5	0	5	3.36
5	500	2.7	1/5	20	20	4.16
10	1000	3	1/5	40	40	5.16
20	2000	3.3	3/5	60	60	5.25
40	4000	3.6	4/5	80	95	5.84
80	**8000**	**3.9**	**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.



LD_{50} of Naja naja

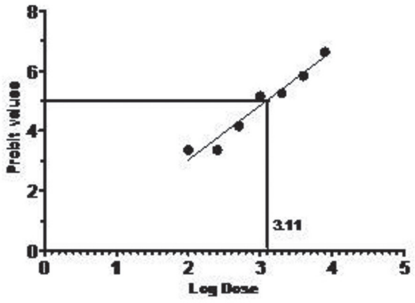
= antilog (log dose)/100

= antilog 1.48/100

= 30.19/100

= 0.301 µg/g

FIGURE 4 - Calculation of lethal dose of *Naja naja* venom.



ED_{50} of Rauvolfia serpentina against Naja naja venom

= antilog (log dose)/100

= antilog 3.11/100

= 1288/100

= 12.88 mg

FIGURE 5 - ED50 of Rauvolfia serpentina against *Naja naja* venon.

DISCUSSION

During the last few years there has been an increasing interest in the study of medicinal plants and their traditional use of different parts of India. In the recent years number of reports on the use of plants in traditional healing by either tribal people or indigenous communities of India is increasing (Namsa *et al*., 2009; Upadhyay, Dhaker, Kumar, 2010). The herbal medicines are mostly administered in the form of juice, decoction, paste or powder, prepared in a crude method from different plant parts such as root, bark, leaves, flowers, fruits, seeds and whole plant (Sarada *et al*., 2008). The aqueous extracts were selected for the study because the 'vishavaidayas' used only the aqueous extracts to treat the ill effects of snake bite. Earlier research on plants as antivenom also supports the use of aqueous extract (Houghton, Osibogun, 1993). Singh (2008) has stated the ethno remedial use of *Rauvolfia serpentina* plant against snake bite. Root decoction is being used as an antidote to snake venom in some tribal rich district of Orissa, India (Behera, Sahoo, Mohapatra, 2007). About 10 mL of root paste is taken orally for management of snake bite by the forest inhabitants of the Daitari range of hills of Orissa, India (Mohapatra, Prusty, Sahoo, 2008). Pattanaik, Reddy and Reddy (2009) have informed the use of this plant (Known as Patalgaruda locally) by the local people of Eastern Ghats, India against snakebite. In our present investigation antivenom potential for *Rauvolfia serpentina* aqueous root extract against *Naja naja venom* was studied by *in vivo and in vitro* methods

TABLE VI - Activities of the compounds identified from the extract of *Rauvolfia serpentina* root by LC-MS analysis (PASS)

SL NO	Compound name	Molecular mass	Molecular formula	Activities on Snake Venom
1	D-Glucuronic acid	194.14	$C_6H_{10}O_7$	Antidote, ATPase inhibitor, L-amino-acid oxidase inhibitor, Acute neurologic disorders treatment Phospholipase A1,A2 inhibitor, 5'-Nucleotidase inhibitor
2	Triacontanol	438.81	$C_{30}H_{62}O$	Antidote, Phospholipase activity, 5'nucleotidase inhibitor
3	Alppha-ionine	192.32	$C_{13}H_{20}O$	ATPase inhibitor.
4	Hydroquinone	110.11	$C_6H_6O_2$	L-aminoacid oxidase inhibitor, 5'nucleotidase inhibitor, ATPase inhibitor
5	Reserpinine	634.74	$C_{22}H_{26}N_2O_4$	Phosphodiesterase inhibitor
6	Reserpine	608.69	$C_{33}H_{40}N_2O_9$	Antidote, Phosphodiesterase inhibitor
7	Ascorbic acid	176.12	$C_6H_8O_6$	ATPaseinhibitor, 5'nucleotidase inhibitor
8	Gallic acid	170.13	$C_7H_6O_5$	Antidote, ATPase inhibitor, venom exonuclease inhibitor, Acute neurological disorder treatment, 5'-Nucleotidase inhibitor
9	Hydrangenol	256.26	$C_{15}H_{12}O_4$	ATPase inhibitor activity, Phospholipase inhibitor
10	Oleic acid	282.47	$C_{18}H_{34}O_2$	L-aminoacid oxidase inhibitor, ATPase inhibitor

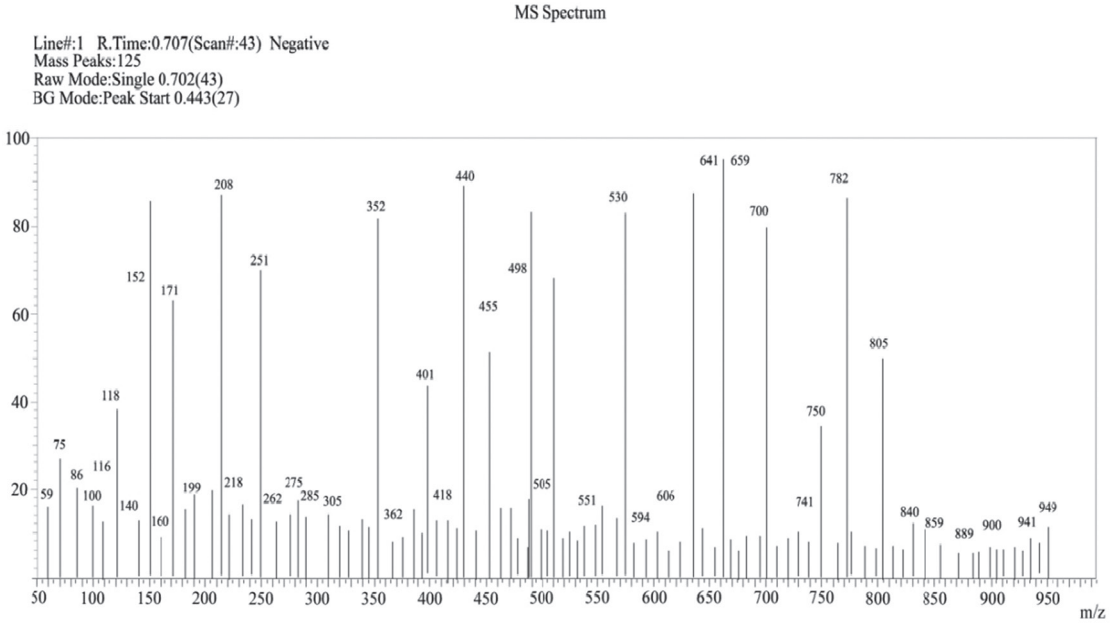


FIGURE 6 - LCMS analysis Rauvolfia serpentina root.

In vitro neutralization assays

Maximum of Acetyl cholinesterase inhibition (60.29%), protease inhibition (50.40%), and ATPase inhibition (58.76 %) was occurred for 300 µg concentration of aqueous plant extract. The inhibition of *Naja naja* venom enzymes by increased amounts of aqueous extract from *Rauvolfia serpentina* was very effective, when the extract was previously mixed with venom. There was a substantial deactivation of Acetyl cholineesterase, Protease, ATPase activities. The studies of Kadiyala (2011) have also reported the inhibition of *Naja naja* venom enzymes by increased amounts of methanol extracts from *Andrographis paniculata*.

In vivo neutralization assays

The plant extract effectively neutralized the *Naja naja* venom induced lethal activity. About 0.14 mg of *Rauvolfia serpentina* plant extract was able to completely neutralize the lethal activity of $2LD_{50}$ of *Naja naja* venom. The alkaloid reserpine inhibits the action of phospholipase- A2 enzyme from *Naja naja* venom. In our study *In vivo* assessment of venom toxicity (LD_{50}) of *Naja naja* venom was assessed by LD_{50} range finding test and the median lethal dose (LD_{50}) assayed using mice (18-20g). LD_{50} of *Naja naja* venom was calculated by Miller and Tainter method and was found to be 0.301 µg/g .Venom neutralizing potency tested (ED_{50}) using *Rauvolfia serpentina* root extract was done by Miller and Tainter method and was found to be 12.88 mg against *Naja naja* venom. In previous report on Rajasree, Singh and Sankar (2013) the ethanol extracts from *Rauvolfia serpentina* plant were tested for antivenom activity against *Naja naja* venom. The plant extract effectively neutralized the *Naja naja* venom induced lethal activity. About 0.14 mg of *Rauvolfia serpentina* plant extract was able to completely neutralize the lethal activity of $2LD_{50}$ of *Naja naja* venom. In another study of James *et al*. (2013) of *in vivo* and *in vitro* neutralizing potential of *Rauvolfia serpentina* plant extract against *Daboia russelli venom,Rauvolfia serpentina* plant extract was *e*ffectively neutralized the venom lethality and effective dose (ED) was found to be 10.99 mg/ 3LD of venom

The tests of 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. There were no signs of gross toxicity, antagonistic pharmacological effects or uncommon behavior.

LCMS analysis of aqueous extract of *Rauvolfia serpentina* root

In the present study the phytochemical profile of aqueous extract from *Rauvolfia serpentina* root were characterized. Various antivenom compounds were detected from LCMS analysis of aqueous root extract which includes D-glucuronic acid, triacontanol, reserpine, gallic acid and oleic acid due to inhibition of various snake venom enzymes activities like phospholipase A_2 inhibition, ATPase inhibition, nucleotidase inhibition, 5'nucleotidase inhibition, L-aminoacid oxidase inhibition, phosphodiesterase inhibition activity and have antidote activity. Active phytochemical compounds like D-glucuronic acid, gallic acid, oleic acid isolated from different plant sources, have already been proven for their anti-venom potential (Butt *et al*., 2015). The alkaloid reserpine inhibits the action of phospholipase- A2 enzyme from *Naja naja* venom. Many plant extracts have been reported to possess a detoxifying effect on snake venoms (Haruna, Choudhury, 1995). The mechanism of action of the plant extracts/plant compounds are still not clear and they may be attributed to the blocking of receptors-structure prone to chemical attack, and may block the active site of the snake venom. Hence, the presence of these anti snake venom compounds in the aqueous extract from *Rauvolfia serpentina* root could have contributed to its efficient antivenom activity.

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