Research Article

In vitro and *in vivo* neutralizing potential of *Alangium salvifolium* extract against Russell's viper (*Daboia russelli*)

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Abstract

Objective: The objective of the work is to determine the Russell viper venom neutralization efficiency of the fraction of methanolic extracts of *Alangium salvifolium by in vitro* and *in vivo* methods. **Material and methods:** In the present study, the anti-snake venom activity was investigated by using different *in vitro* methods *viz*., phospholipase inhibition activity and Neutralization of Coagulant activity of venom. The ethyl acetate fraction was subjected to *in vivo* study by Neutralization of lethal effect (ED_{50}) of Venom, Neutralization of haemorrhagic activity and Neutralization of mast cell degranulation. **Results and conclusion:** Different doses of fractions were effective against Russell's viper venom in a concentration dependant manner. But it was found that ethyl acetate fraction at a dose of 400 µg inhibited greater than half of the halos 4.33 mm (68.21%) in phospholipase inhibition activityand showed a high significant ($P<0.001$) reduction in the coagulant activity with time period of 144.33±1.51s (77.73%). Ethyl acetate fraction showed potent snake venom neutralizing capacity against Russell viper venom in Albino wistar rats and swiss albino mice in dose dependent manner.100% neutralization of median lethal dose (LD_{50}) 0.6µg/g of venom was observed in 500mg/kg of ethyl acetate fraction treated animals. Ethyl acetate fraction (500 mg/kg) showed significant decrease in haemorrhagic lesions (81% inhibition) and inhibit venom induced mast cell degranulation (49.52% protection). The results are comparable to Standard antisnake venom (ASV).

Keywords: *Alangium salvifolium,* anti-snake venom, Russell's viper venom

Introduction

Envenoming resulting from snake bite is a major public health concern, especially in rural population of tropical and subtropical countries. Snake bite is a major health hazard that leads to high mortality and great suffering in victims. It has been reported that there are 5 million snake bites with 2,50,000 envenomation and around 1,25,000 fatalities annually in the world in which India have the highest number of snake bites and deaths per year (Bhardwaj and Sokhey, 1998).

India has reported highest number of snake bite cases and fatalities estimating 35,000 – 50,000 death every year (Bawaskar, 2004).Although antiserum is an effective antidote, it quite often produces severe allergic reactions and side effects. Anti-serum requires refrigeration; it is expensive and often not available in rural areas. Antivenom therapy has several adverse effects on various organs of human body hence it is necessary to find alternative to eliminates this side effects. Thus the finding of antitoxin against venom is of significant concern. The knowledge of herbal drugs used in snakebite is scanty and hence discrete (Emmanuel, and Selvanayagam, 1994).

In Ancient time, plants based remedies is implicated for the snake bites treatments. Different parts of *Alangium salvifolium* are used for a wide range of diseases. The major phytochemical constitutes of the plant are alangine A and B, alangicine, markindine, lamarckinine and emetine (Anonymous, 1952). Root bark is emetic, febrifuge, purgative, anthelmintic, diaphoretic, antipyretic and useful in fever, snake bite and piles. Its root is used as diuretic, astringent and antidote for several poisons (Vaidya and Bothr, 2014).Roots are also used locally as remedy for

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snakebite, scorpion bite as well as for dog bite. Bark of this tree is bitter, purgative, anthelmintic, astringent, pungent, efficacious in leprosy, has emetic properties and useful in fever, skin diseases and as a purgative, anti-poisonous against rat, snake and insect bites, antipyretic, anti-inflammatory, analgesic, diuretic, anthelmintic, antidiarrhoeal (Kirtikar and Basu, 1994; Yusuf et al., 1994; Sharma, 1996) Successive methanolic extract of *Alangium salvifolium* showed potent antisnake venom activity (Movalia and Dudharejiya, 2018). The present investigation explored the *D. russelli* venom neutralizing activity of methanolic extract fractions of *Alangium salvifolium* by *in vitro* and *in vivo* models.

Materials and methods

Collection and authentication of plant material

Roots of *Alangium salvifolium* were collected from Munjaka, kalawad road, Rajkot and the herbarium was preserved at S. J. Thakkar Pharmacy College, Rajkot for future reference. Roots were cut in thin-round slices and dried under sun. The dried plant material was then subjected to coarse powder and passed it from sieve no. 40 # and stocked in air tight bottle for further studies.

Successive methanolic extract was prepared by soxhlet extraction and was subjected for fractionation with different solvent from non polar to polar that was petroleum ether (PE), chloroform, ethyl acetate (EA) respectively.

Preliminary chemical tests were carried out for different fraction to identify the presence of Alkaloids, Flavonoids, Glycoside, Tannins, Steroid, Carbohydrates, Protein and Amino acid (Kokate, 1991, Harborne, 1998).

Venom

Russell viper venom in lyophilized form was procured from Irula Snake Catcher's I.C.S. Ltd., Vadanemmeli Village, Kancheepuram Dist., Tamil Nadu, and India and was stored at 4^0C.

Prior utilisation, the venom solution was prepared in saline. Centrifugation was carried out at 2000 rpm for 10 min and for antivenom studies, the supernatant used.

Antisnake Venom serum

Antisnake venom serum was obtained from, Junagadh, Gujarat, India.

Animals

Swiss albino mice (18-20 gm.) and albino wistar rats (180-200 gm.) were used (of either sex) for work. Standard condition (12hr light/dark cycle, 24.c, 35 to 60% humidity) were provide to keep alive and provided free excess to purified drinking water and pelleted diet ad libitum, unless specified. Experimental protocol was approved by IAEC and all experimental procedure was followed according to protocol approved by IAEC committee and guideline of CPCSEA.

In vitro antisnake venom activity

Determination of Neutralization of PLA_2 activity

a) Determination of Minimum Haemolytic Dose (MHD) of Russell's viper venom

In order to evaluate the phospholipase A_2 (PLA_2) activity of Russell's viper venom, the indirect haemolytic activity was assayed as described by Gutiérrez et al (Gutierrez et al., 1988). 300 µl of packed sheep erythrocytes was held four times with saline solution, 300µl of 1:3 egg yolk solution in saline solution and 240µl of 0.01 M $CaCl_2$ solution was added to 25 ml of 1% (w/v) of agar at 50 ºC & dissolved in PBS pH 7.2. The mixture was applied to Petri dish and allowed to gel. Then, 3 mm diameter wells was made and filled with 15µl of solutions containing different concentrations of venom (from 1 to 50µg). After 20 hr of incubation at 37ºC, the diameters of haemolytic halos were measured. Control wells were containing 15µl of Phosphate buffer of pH 7.2. The minimum haemolytic dose (MHD) was defined as the amount of venom that induced a haemolytic halo of 11-mm diameter.

b) Neutralization of Phospholipase activity at Minimum Haemolytic Dose

Each fraction of methanolic extract was evaluated for neutralization of phospholipase A2 activity (indirect haemolytic activity). A sample of constant amount of venom (1 MHD) was incubated with different amount of fraction for 30 min at 37ºC. Then aliquots of 14µl of the mixtures (venom + fraction) was added to wells in agarose egg yolk-sheep-erythrocytes gel plates and incubated at 37ºC for 20 hr. Control samples was contained venom (1 MHD) without extract. The standard reference group of rats was administered with antivenom after administration of venom. Plates was incubated at 37ºC for 20 hr. Neutralization was expressed as the % Inhibition that reduced 50% the diameter of the haemolytic halo when compared to the effect induced by venom alone.

Determination of Neutralization of Coagulant activity of Russell's viper venom

a) Determination of Minimum Coagulant dose plasma (MCD-P) of Russell's viper venom

The procoagulant activity was done according to the method described by Theakston and Reid (Theakston et al., 1983,) modified by Laing *et al*. (Laing et al., 1992). It was carried out by taking various concentration of venom (1-150 µg), dissolved in 100 µl of PBS of pH 7.2 and to this 300 µl of human citrated plasma was added, to this 100µl of 0.25M $CaCl_2$ was added and the clotting time was observed every 15 sec. by gentle tilting of experimental tube until

coagulation occurs. Plasma incubated with PBS alone served as control. MCD-P was defined as the least amount of venom that clots plasma in 60 sec at 37^{0}C.

b) Neutralization of coagulation activity

The neutralization of coagulation activity of Russell's viper venom by each fraction was done by taking constant amount of venom (2 MCD-P) which was mixed with various concentrations (50-400µg) of fractions. The mixtures was incubated for 30 min at 37°C and then 100µl of mixture was added to 300 µl of citrated plasma, the plasma was re-calcified with the addition of 100 µl of 0.25 M CaCl2 and the clotting times was recorded by gentle tilting at every 15 s till coagulation occurred.

***In vivo* study**

1) Determination of Median Lethal Dose of venom (MLD)

The toxicity studies were carried out in mice weighing 18-20gm of either sex to find out the median lethal dose. The MLD was determined by administering various concentration of Russell's viper venom in a volume of 200µl physiological saline, in four groups of mice containing six in each.

Initially doses of 0.2µg/g, 0.4µg/g, 0.6 µg/g and 0.8µg/g venom were administered by i.p. and animals in each group were observed for mortality up to 72 hours. A concentration producing 50% mortality was used as a median lethal dose, selected to induce lethality in animal for determination of ED_{50} (Pithayanukula et al., 2005).

2) Determination of Neutralization of lethal effect (ED_{50}) of Venom

The ability of test drugs to inhibit lethal action of the venom was be assessed by i.p. administration of LD_{50} of venom into groups of mice (n=6), followed after 5 min by i.p. administration of250mg/kg and 500 mg/kg of ethyl acetate fraction (Pithayanukula et al., 2005). The standard reference group of mice was administered with antivenom after administration of LD_{50} of venom. The number of dead mice in each group was counted.

Treatment in each group was given as follow:

Group 1: Control- Vehicle (1% CMC)

Group 2: ASV Standard - Antisnake venom serum

Group 3: EAAS 250mg/kg - Ethyl acetate fraction of *A. salvifolium* (250 mg /kg)

Group 4: EAAS 500mg/kg - Ethyl acetate fraction of *A. salvifolium* (500 mg /kg)

3) Determination of Neutralization of haemorrhagic activity

The minimum hemorrhagic dose (MHD), i.e. the minimum concentration of venom which when injected intradermal into rats produces a hemorrhagic lesion of 10 mm diameter after 24 h of injection, was determined (Kondo, 1960). The ability of test drugs to inhibit the hemorrhagic action of venom was assessed by intradermal administration of MHD of venom into the shaved dorsal skin of the groups of rats (n=6), followed after 5 min by i.p. administration of different doses of EAAS. The standard reference group of rats was administered antivenom after administration of MHD of venom.

Treatment in each group was given as follow:

Group 1: Control- Vehicle (1% CMC)

Group 2: ASV Standard - Antisnake venom serum

Group 3: EAAS 250mg/kg - Ethyl acetate fraction of *A. salvifolium* (250 mg /kg)

Group 4: EAAS 500mg/kg - Ethyl acetate fraction of *A. salvifolium* (500 mg /kg)

4) Determination of Neutralization of mast cell degranulation

The *in vitro* method (Kannappa et al., 1993) was modified for *in vivo* determination of mast cell degranulation.The ability of test drugs to inhibit venom induced mast cell degranulation was assessed by the nonlethal dose of viper Russell venom injected (i.p.) to groups of rats, followed after 5 min by administration of different i.p. doses of EAAS. The standard reference group of rats was administered with antivenom after administration of nonlethal of venom (Table 3). After 24 hour the rats was sacrificed and mesentery cut into small bits about 1 cm. The mesentery piece was washed with tyrode solution and spread over glass slide. The mast cells were stained with 1% toluidine blue and count under a high objective field and the percentage of degranulation was noted.

Treatment in each group was given as follow:

Group 1: Control- Vehicle (1% CMC)

Group 2: ASV Standard - Antisnake venom serum

Group 3: EAAS 250mg/kg - Ethyl acetate fraction of *A. salvifolium* (250 mg /kg)

Group 4: EAAS 500mg/kg - Ethyl acetate fraction of *A. salvifolium* (500 mg /kg)

Statistical analysis

After completion of the study values were expressed as Mean ± S.E.M. and analysed by one way ANOVA followed by Brown-forsythe multiple range test apply using graph pad software and $p<0.05$ was considered as statistically significant activity as compare to standard group.

Results and discussion

The results of phytochemical screening of fractions (Table 1) reveal the presence of alkaloid, saponins, tannins, phenolics, flavanoids, steroids and terpenoids.

Table 1. Qualitative chemical tests of fractions of roots of *A. Salvifolium*

Sr. No.	Test for	EE	PE	Chloroform	Methanol
1	Carbohydrates	+	-	+	+
2	Proteins	-	-	-	-
3	Steroids	-	+	-	-
4	Glycosides	-	-	-	-
5	Flavonoids	+	-	+	+
6	Alkaloids	+	-	+	+
7	Tannins	+	-	+	+

Table 2. The effect of ASV on Russell's viper venom (PLA_2) induced haemolysis on sheep RBC's gel plate

Groups	Dose of venom	Dose of extract	Haemolytic halos (mm)	% Inhibition
Normal	PBS	-	0.9 ± 0.12	-
Venom	12 µg	-	13.3 ± 0.45#	-
ASV	12 µg	-	3.93 ±0.11**	71.14

All values are in Mean ± SEM, n=3 in each group; # significantly different from normal control ($p < 0.001$); ** is significantly different from disease control ($p < 0.001$)

In vitro antisnake venom activity

Determination of Neutralization of PLA_2 activity

About 12 µg of Russell's viper venom produced 12 mm diameter haemolytic halo (Table 2). This indicates that lyses of sheep RBC's occurs due to enzymes (phospholipase A_2) present in Russell's viper venoms.

Different doses of fraction were effectively inhibiting phospholipase A_2 dependant haemolysis of sheep RBC's in a dose dependant manner. But it was come to conclusion that ethyl acetate fraction showed potent inhibition activity as compare to other fraction.

It was found that 400µg (Table 3) of Ethyl acetate fraction inhibited greater than half of the halos 4.33 mm (68.21%). The standard ASV produces 71.14% inhibition of halos produced by the phospholipase A_2 dependent haemolysis of sheep RBC's induced by Russell's viper venom.

Most of the snake venoms include phospholipase yet haemolysin which work on the membrane related phospholipids releasing lysolecithin. Lysolecithin work on the membrane of HRBC leads to haemolysis. Inhibition of haemolysis by venom by fractions might be due to stabilization of proteins in the membrane of HRBC (Anas, 2010).

Determination of neutralization of coagulant activity of Russell's viper venom

MCD-P of Russell's viper venom was found to be 6 µg. Normal coagulation produced (Plasma + PBS + $CaCl_2$) at

Table 3. The effect of fraction on Russell's viper venom (PLA_2) induced haemolysis on sheep RBC's gel plate

Concentration (µg)	% Inhibition			
	PE(AS)	Chloroform (AS)	EA (AS)	Methanol (AS)
50	21.17	22.73	29.05	21.17
100	26.98	28.60	37.40	29.09
150	35.94	33.49	41.80	34.47
200	40.34	40.09	49.63	44.74
250	42.29	47.67	54.27	52.94
300	46.94	57.94	61.61	57.94
400	54.27	64.54	68.21	63.83

All values are in Mean ± SEM, n=3 in each group; # significantly different from normal control ($p < 0.001$); ** is significantly different from disease control ($p < 0.001$)

Table 4. The effect of ASV on neutralization of coagulation activity of Russell's viper venom

Groups	Dose of Venom	Dose of Extract	Clotting formation time (Sec)	% Neutralization
Normal	-	-	185.66 ± 1.52	-
Venom	12 µg	-	14.33 ± 0.87#	-
ASV	12 µg	-	144.83 ± 0.75**	78.00%

Table 5. The effect of fraction on neutralization of coagulation activity of Russell's viper venom

Concentration (µg)	% Inhibition			
	PE	Chloroform	EA	Methanol
50	22.26	31.23	35.90	26.03
100	30.52	44.52	47.57	33.75
150	36.26	50.62	51.34	38.59
200	44.34	57.80	57.80	48.11
250	49.19	62.83	64.81	55.11
300	55.65	68.58	72.89	62.83
400	78.00	73.96	77.73	68.58

All values are in Mean ± SEM, n=3 in each group; # Significantly different from normal control ($p < 0.001$); ** is significantly different from disease control ($p < 0.001$)

Table 6. *In vivo* determination of LD_{50} of Russell's viper venom

Groups	Dose in µg/g	Total animals	Mortality	% Mortality
1	0.2 µg/g	6	0	0 %
2	0.4 µg/g	6	0	0 %
3	0.6 µg/g	6	3	50 %
4	0.8 µg/g	6	5	83.33

Table 7. Neutralization studies of Russell's viper venom by Ethyl acetate fraction of AS

Groups	Treatment	Venom (µg/g)	No. of Animals	Deaths	% Neutralization
1	EA(250 mg/kg)	0.6	6	2	67%
2	EA(500 mg/kg)	0.6	6	0	100

Table 8. Neutralization of haemorrhagic activity of Russell's viper venom by Ethyl acetate fraction

Groups	Treatment	Haemorrhagic lesion (mm)	% Inhibition
1	Venom	12.48 ± 0.33	--
2	ASV	5.16 ± 0.56**	58.65
3	EA(250mg/kg)	7.78 ±0.23	37.65
4	EA(500mg/kg)	5.01±0.41	59.81

All values are in Mean ± SEM, n=6 in each group; ** is significantly different from Disease control ($p < 0.001$)

time period of 185.66 ±1.52s. Ethyl acetate fraction of methanolic extract of at a dose of 400 µg showed a high significant ($P<0.001$) reduction in the coagulant activity with time period of 144.33±1.51s (77.73%). ASV showed inhibition at 144.83± 0.75 (78.00%) against Russell's viper venom. All the data were summarised in table 4 and 5. So, from the results of *in vitro* study, the most active ethyl acetate fraction (EA) was subjected for *in vivo* study.

In vivo study

1) Determination of Median Lethal Dose of venom (MLD):

LD_{50} of Russell's viper venom was found to be 0.6 µg/g in Swiss albino mice (Table 6).

2) Determination of Neutralization of lethal effect (ED_{50}) of Venom

Russell's viper venom induced lethality was significantly antagonized by ethyl acetate fraction. 100% neutralization of median lethal dose (LD_{50}) 0.6µg/g was observed in 500mg/kg of ethyl acetate fraction treated animals. Results are depicted in table 7.

3) Determination of Neutralization of haemorrhagic activity

About 0.6µg/g of venom induced 12.48 ± 0.33 mm diameter haemorrhagic lesions into the shaved dorsal skin of the rats after 24hr and it was considered as MHD of Russell's viper venom.

Table 9. Neutralization of mast cell degranulation of Russell's viper venom by EAAS

Groups	Treatment	Total mast cell	Degranulated mast cell	% Inhibition
1	Normal	100	15 ± 0.55	--
2	Venom	100	85 ± 2.05#	--
3	ASV	100	31 ±0.98**	64.00
4	AS (250mg/kg)	100	47.83 ± 2.09	44.91
5	AS(500mg/kg)	100	43.83 ± 2.55	49.52

All values are in Mean ± SEM, n=6in each group; # significantly different from disease control ($p < 0.001$); ** is significantly different from normal control ($p < 0.0$)

Animals pre-treated with standard ASV and administration of lower and higher dose of EA (Table 8) challenging to MHD of venom showed significant decrease in haemorrhagic lesions ($P < 0.001$).

4) Determination of Neutralization of mast cell degranulation

There was a significant increase in de-granulated mast cell count (85 ± 2.05) in venom treated group, while animals containing treatment of EA, de-granulated mast cell count was significantly decreased.

Standard ASV showed 64.00% protection (31 ±0.98) and the animals treated with 500 mg/kg dose of EA showed 49.52% protection compare to venom treated mast cell solution. A result shows that higher dose showed nearer beneficial effect as standard treatment ASV (Table 9).

Conclusion

Thus, it can be concluded from the study that *A. Salvifoliu*m roots have significant antisnake venom activity. Ethyl acetate fraction of Methanolic extract showed better anti-venom activity as compared to other fractions and comparable to Antivenom. Hence, these findings support the fact that this plant could be useful in herbal healthcare system against snake bite. However further elaborative studies are needed to isolate and characterize the active constituents and mechanism of venom inhibition.

Conflicts of interest

We declared that we do not have any conflicts of interest in this research.

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