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ANTI-SNAKE VENOM ACTIVITY OF DIFFERENT EXTRACTS OF *POUZOLZIA INDICA* AGAINST RUSSEL VIPER VENOM

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ABSTRACT: Snakebite is an important cause of morbidity and mortality and is one of the major health problems in India. Russell's viper or daboia (*Viper russelli)* appears to be the commonest cause of fatal snakebite in Southern India, Pakistan, Bangladesh, Sri Lanka, Burma and Thailand. Intravenous administration of antibothropic antivenom neutralizes the systemic actions, but it is of little effect on the reversal of local symptoms and often induces adverse reactions, a context that drives the search for complementary treatments for snakebite accidents. Vegetable extracts with a range of antiophidian activities constitute an excellent alternative. In this study, we investigated the anti-venom effects of different extracts of *Pouzolzia indica.* The alcoholic and aqueous extracts of *P. indica* showed potent snake venom neutralizing capacity against Russell viper venom.
Keywords: Antiophidic plants; Anti-hemorrhagic; anti-snake venom; *Pouzolzia indica.*

INTRODUCTION

Snakebite is an important cause of morbidity and mortality and is one of the major health problems in India[1]. Russell's viper or daboia (*Viper russelli)* appears to be the commonest cause of fatal snakebite in Southern India, Pakistan, Bangladesh, Sri Lanka, Burma and Thailand[2]. *Viper russelli russelli* (Indian subspecies of Russell's viper) is one of the four-major classes of snakes that cause death in the Indian sub-continent[3]. Antivenom has been used for decades and is only treatment for the snakebites. However antivenom therapy has several adverse effects on various organs of human body because of the administration of foreign proteins, sensitization to horse serum and the presence of immune complexes. There are two primary risks associated with antivenom therapy viz. acute anaphylaxis or anaphylactic reactions (mild to severe) which occur within one hour of antivenom administration[4-6] are characterized by urticaria, wheezing, tachycardia, hypotension, nausea, vomiting, diarrhea, angioedema, respiratory failure, upper airway edema, shock and death[5] and serum sickness which can occur between 5 to 24 days after antivenom therapy[2]; is a type III hypersensitivity response which develops due to the formation of

antigen-antibody complexes[7]. Cases usually present with fever, itching, urticaria, arthralgia, and generally respond to therapy with antihistamines, nonsteroidal antiinflammatory agents and steroids[5]. In severe cases, neurotoxic symptoms may be present[2]. All these hazards and drawbacks necessitate the search for antidotes without these defects.

Plant extracts contain a large diversity of chemical compounds displaying several pharmacological activities. Many herbs have been used traditionally for the treatments of snakebites. The tribals of various regions have found their own remedies and treat snakebite. e.g. ***Achyrathus aspera, Allium sativum, Azadirachta indica, Calotropis gigantiea, Rauvolfia serpentina*** are frequently used by Bhoxas of Nainital and other tribal and rural population. In addition, ***Acorus calamus, Alternanthera sessilis, Anogeissus latifolia, Hemidesmus inidcus, Leucas cephalotes*** etc are also widely used as snake bite remedies. The plant ***Pouzolzia indica*** claimed to be useful in treating snake poison in the Indian system of medicine. The present study evaluated the potential anti-venom effect of different extracts of ***Pouzolzia indica*** by using *in vitro* and *in vivo* methods**.**

EXPERIMENTAL

Collection of the plant material:

The aerial parts of ***Pouzolzia indica*** (Family: Urticaceae) were collected from Manipal, Udupi District, Karnataka, India, in the month of September-November 2006 and authenticated by botanist Dr. Gopalakrishna Bhat, Professor of Botany, Poorna Prajna College, Udupi, India. A herbarium specimen bearing voucher No. PP. (555) has been deposited in the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal University.

Preparation of successive solvent extracts[8-10]:

a) Petroleum ether extract:

The coarsely powdered shade dried leaves of *P. indica* (2 kg.) was exhaustively extracted with petroleum ether by hot extraction process (Soxhlet). After completion of extraction, the solvent was removed by distillation, concentrated and then stored in a dessicator until further use.

b) Benzene extract:

The marc left after petroleum ether extraction was dried and extracted with benzene by hot extraction process (Soxhlet). After completion of extraction, the solvent was removed by distillation, concentrated and then stored in a dessicator until further use.

c) Chloroform extract:

The marc left after benzene extraction was dried and extracted with chloroform by hot extraction process (Soxhlet). After completion of extraction, the solvent was removed by distillation, concentrated and then stored in a dessicator until further use.

d) Acetone extract:

The marc left after chloroform extraction was dried and extracted with acetone by hot extraction process (Soxhlet). After completion of extraction, the solvent was removed by distillation, concentrated and then stored in a dessicator until further use.

e) Ethanolic extract:

The marc left after acetone extraction was dried and extracted with (95%) ethanol by hot extraction process (Soxhlet). After completion of extraction, the solvent was removed by distillation, concentrated and then stored in a dessicator until further use.

f) Aqueous extract:

The marc left after ethanol extraction was dried and extracted with chloroform: water (1:99) by maceration process for 7 days. After completion of extraction, the solvent was removed by distillation, concentrated and then stored in a dessicator until further use.

The various extracts obtained by successive solvent extraction were divided in to two portion for anti-snake venom studied and phytochemical investigations.

CHEMICAL ANALYSIS OF DIFFERENT EXTRACTS[8-10]:

Different chemical tests were carried out for different extracts of *P. indica* to identify the presence of various chemical constituents like alkaloids, saponins, carbohydrates, glycosides, steroids, fixed oils and fats, tannins and phenolic compounds, proteins and amino acids, gums and mucilage and flavonoids.

VENOM:

The lyophilized snake venom *Viper russelli* was obtained from Irula Snake Catcher's I.C.S. Ltd., Vadanemmeli Village, Kancheepuram, Tamil Nadu, India and was preserved at 4^0 C. Before use the venom was dissolved in saline, centrifuged at 2000 rpm for 10 min and the supernatant used for anti venom studies. Venom concentration was expressed in terms of dry weight.

SNAKE VENOM ANTISERUM:

Snake venom antiserum (as reference serum) was obtained from Kasturba Hospital Pharmacy, Manipal India.

IN VITRO ANTISNAKE VENOM ACTIVITY OF DIFFERENT EXTRACTS OF *P. INDICA:*

Preliminary in vitro anti-snake venom activity ***viz.,*** Platelet aggregation, phospholipase inhibition activity were carried out for the various extracts of *P. indica* to select the most active promising extract for further study.

ADP induced platelet aggregation[11]:

The reaction mixture contains different concentration of drug extract 0.5 ml (160, 320, 640 µg/ml) venom solution 0.5 ml (200 µg/ml) and platelet rich plasma 0.5 ml. These reaction mixtures was maintained at

37^0C and kept for 2 min with constant stirring, 0.5 ml of ADP solution was added and incubated for 4 mins and absorbance was measured at 414 nm.
ADP induced platelet aggregation was calculated by following formula:

$$\% \text{ ADP induced platelet aggregation} = \frac{(T_1-T_2)-(T_1-T_3)}{(T_1-T_2)} \times 100$$

Where, T_1= ADP + Platelet, T_2= ADP +Venom +Platelet and T_3= ADP +Venom +Platelet +Plant extract.

Phospholipase inhibition activity[11]:
A mixture contains 50 mg of lecithin (dissolved in 25 ml of diethyl ether), 1 ml of drug solution (160, 320 and 640 µg/ml), 1 ml of venom solution (400 µg/ml), and 0.1 ml of 5% of $CaCl_2$ solution were added and the reaction vessel was swirled or shaken until the reaction mixture becomes homogenous. The reaction was allowed to proceed under room temperature for 4 hr. At the end of incubation period 25 ml of alcohol and 0.3 ml of cresol red solution were added and the solution titrated with 0.02 N methanolic NaOH. A blank performed was prepared by addition of alcohol, venom and $CaCl_2$ to the ether in that order and was titrated immediately. The hydrolysis capacity of PAL exhibited by reacting 400 µg/ml of venom with 50mg lecithin was considered as 100% phospholipase activity and served as control.

Selection of active extract:
Based on the results of preliminary *in vitro* antisnake venom studies *viz.,* platelet aggregation and phospholipase inhibition activity of the different successive solvent extracts screened, the ethanolic and aqueous extracts were found to possess most significant activity. Hence, these two extract (EtOH and aqueous) were selected for *in vivo* antisnake venom studies.

***IN VIVO* ACTIVITY:**

Animals:
Healthy adult Wistar albino rats weighing about 200-250 g and Swiss albino mice; individually in polypropylene cages, maintained under standard conditions (12 h light and 12 h dark cycle; 25± 30^oC; 35 - 60% humidity). The animals were fed with standard rat pellet diet (Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*. The study was approved by the Institutional Animal Ethical Committee of KMC, Manipal, India (IAEC/KMC/07/2007 -2008).

Acute toxicity study[12]:
Acute toxicity studies were conducted to determine the safe dose as per OECD guidelines. Drugs were administered orally to overnight fasted animals. After administration the animals were observed continuously for 1 hour, frequently for the next four hours, and then after 24 hours.
After administration, Irwin's test was conducted, where the animals were observed for gross behavioral changes. For this, the following check list was employed.
i) Behavioral profile
Awareness: alertness, visual placing, stereotypy, passivity.
Mood: grooming, restlessness, irritability, fearfulness.
ii) Neurological profile
Motor activity: Spontaneous activities, reactivity, touch response, pain response, startle response, tremor, gait, grip strength, pinna reflex, corneal reflex.
iii) Autonomic profile:
Writhing, defecation, urination, pilo erection, heart rate, respiratory rate.

Selection of doses:
The doses selected for the extracts were about 1/10th of the maximum tolerated safe dose found from acute toxicity studies. They were administered once daily by oral route.
a) Alcoholic extract: Dose A –250 mg/kg, Dose B - 500 mg/kg
b) Aqueous extract: Dose A - 250 mg/kg, Dose B - 500 mg/kg

Neutralization potential of different extracts of *Pouzolzia indica* on lethal venom effect:
The toxicity of venom was assessed in Swiss albino mice (18-22g) and Wistar rats (200-250g) by i.p. administration of different concentrations of venom dissolved in 0.2 ml of physiological saline to different groups (n =10). The median lethal dose (LD_{50}) and lethal dose (LD_{100}) of venom was determined 24h later by the method reported earlier[13]. The neutralizing potency of the different aerial part of plant extracts (alcoholic and aqueous) was assessed by i.p. administration of LD_{100} dose of venom into groups of mice (n=10), and LD50 dose of venom in to rat (n=24 in control, n=16 in treated) followed by oral dose administration of different doses of the plant extracts. The standard reference group i.e. snakevenom anti serum was administered after administration of LD_{100} and LD_{50} dose of venom. The experiment was performed in duplicate.

Neutralization potential of alcoholic and aqueous extract of *P. indica* on haemorrhagic activity and necrotic activity:
The minimum haemorrhagic dose (MHD) is the least amount of venom (µg/dry weight) which when injected intradermally into rats results in a haemorrhagic lesion of 10mm diameter 24h later[14] and the minimum necrotizing dose (MND) is the least amount of venom (µg/dry weight) which when injected intradermally into rats, results in a necrotic lesion of 5mm diameter,

3 days later[13]. The MHD and MND dose, intradermally injected into the shaved dorsal skin of the rats was followed after 5 min by i.p administration of the different doses of alcoholic and aqueous extract. The experiment was performed in duplicate.

Neutralization potential of alcoholic and aqueous extract of *P. indica* on mast cell degranulation:

The LD50 dose of *Viper russelli* venom injected (i.p) to groups of rats; was followed after 5 min by administration of different i.p. doses of leaf extracts. After 1st and 5th day the rats were sacrificed and mesentery cut into small bits of about 1 cm. These were carefully washed with tyrode solution and spread over glass slides. The mast cells, stained with 1% toluidine blue were counted under a high power objective field and the percentage of degranulation was noted[15].

Lipid peroxidation products- TBARS:

The pancreas of the different groups of animals was dissected out carefully, washed in normal saline and TBARS (Thiobarbituric acid reactive substances) levels were estimated[16].

RESULTS AND DISCUSSION

Six crude extracts of *P. indica* were investigated for their potential anti snake venom activity. The average value of extractive is shown in table 1.

The obtained extracts were subjected to phytochemical screening for its constituents by standard methods and the results were tabulated in table 2. Preliminary phytochemical studies revealed the presence of alkaloids, carbohydrate, Phenolic compounds, gum, mucilage and tannins.

The results of preliminary *in vitro* antisnake venom studies *viz.,* platelet aggregation and phospholipase inhibition activity of the different successive solvent extracts are shown in table 3. The PAL A_2 present in the viper venom can causes inhibition of ADP induced platelet aggregation. The alcoholic and aqueous extracts of *P. indica* significantly decreased the inhibition of platelet aggregation. In our study, the alcoholic and aqueous extracts of *P. indica*, significantly inhibited the PAL enzyme activity may be by its iteration with PAL A_2 and thereby preventing hydrolysis of lecithin.

It revealed the ethanolic and aqueous extracts were found to possess most significant activity. Hence, these two extract (EtOH and aqueous) were selected for more detailed in vivo antisnake venom studies.

Administration of (alcoholic and aqueous extracts) of *P. indica* at two dose levels i.e. 250, 500mg/kg body weight in mice, and rats show significant antisnake venom activity. However the alcoholic extract showed significant antivenom activity at a dose level of 500mg/kg-body weight. The higher activity of the alcoholic extract suggests that some alcoholic soluble chemical compounds present in the aerial extract may be responsible for the activity. The neutralization potential of alcoholic and aqueous extracts of *P. indica* on the %increase in survival rate in envenomed mice (LD_{100}) and rat (LD_{50}) is shown in table 4 and 5.

According to the WHO, the anti snake venom possessing compound should be tested regarding its capacity to neutralize venom effects such as lethality, haemorrhagic and necrotizing effects[13]. Most venom possesses the ability to cause local necrosis and haemorrhage when introduced intradermally. Hence, the minimum necrotizing dose and minimum haemorrhagic dose estimation proves a reasonable test for assessing the antivenom activity. Intravascular haemolysis may contribute to the development of acute tubular necrosis and bilateral cortical necrosis in victims of Russell's viper bite. Haemorrhagins causes death due to bleeding from vital organs by damaging vascular endothelium[17]. The alcoholic and aqueous extracts of *P. indica* were found to significantly reduce the viper venom induced haemorrhagic and necrotic lesions (Table 6).

The release of viper venom causes an enormous amount of histamine into circulation by mast cell degranulation. The released substances could also add to the various toxic signs and in fact may be responsible for some of the toxicity such as anaphylaxis[18]. Mast cells are a rich source of mediators like histamine and platelet activating factors (PAF). These two mediators are implicated in several hypersensitive anaphylactic reactions and in the development of various toxic signs after snakebite. Phospholipase A2 present in viper venom might release a large amount of platelet activating factor in addition to histamine and other anaphylactic mediators from mast cells[15]. The result of inhibition of degranulating effect of venom by the alcoholic and aqueous extract is shown in table 7. It may be possible due to interaction with phospholipase A2 and by prevention of the release of platelet activating factor and histamine from mast cells.

It has been reported that anti oxidant activity has been one of the mechanisms of venom inactivation and inhibition[19]. In our study, TBARS levels were significantly reduced by the extracts (Table 8). Hence it may be postulated that the anti snake venom activity of these extracts may at least be partly mediated though it's *in vivo* anti oxidant property.

The results of present study confirm the potent snake venom neutralizing capacity of the alcoholic and aqueous extracts of *P. indica* against Russell viper venom. Further study on isolation of active constituents from these extracts and its anti snake venom activity could lead to the development of a new chemical antidote for snake envenoming.

Table 1: Average value of extractive of different extracts of *P. indica*

S.No.	Solvent	Colour and Consistency	Average value of Extractive (w/w)
1.	Petroleum Ether (60-80°)	Greenish black (sticky solid)	2.12 %
2.	Benzene	Greenish black (sticky solid)	0.76 %
3.	Chloroform	Dark green (sticky solid)	0.52 %
4.	Acetone	Reddish brown (sticky solid)	1.3 %
5.	Ethanol (95%)	Reddish brown (sticky semi solid)	6.3 %
6.	Chloroform water	Dark brown (non-sticky solid)	2.1 %

Table 2: Phytochemical screening of *P. indica*

Test	Acetone extract	Ethanol extract	Water extract
Alkaloids	+	+	-
Carbohydrates	+	+	+
Phytosterols	-	-	-
Fixed oils and fats	-	-	-
Saponins	-	-	-
Phenolic compounds and tannins	+	+	-
Gums and Mucilage	-	-	+

Table 3: *in vitro* antisnake venom study of *P. indica*

S. No.	Drug	Conc. µg/ml	%Platelet aggregation	PAL enzyme activity (%)	PAL inhibition activity (%)
1.	Control			100	0.0
3.	Acetone extract	160	6.8	100	0.0
		320	35.73	89.00	11.00
		640	48.08	89.00	11.00
4.	EtOH extract	160	61.85	84.21	15.79
		320	72.95	68.42	31.58
		640	88.90	63.15	36.58
5.	Aqueous extract	160	43.95	89.00	11.00
		320	61.45	73.68	26.32
		640	84.12	68.42	31.58

Table 4: Neutralization potential of alcoholic and aqueous extracts of *P. indica* on the %increase in survival rate in envenomed mice (LD_{100})

Treatment	N	% Survival	%↑ in survival as compare to control
Control (1%CMC)	10	0	00
Standard (antivenom)	10	83.33	100
Aqueous extract (250mg/kg)	10	33.33	33.33
Aqueous extract (500mg/kg)	10	50	50
Alcoholic extract (250mg/kg)	10	50	50
Alcoholic extract (500mg/kg)	10	66.67	66.67

Table 5: Neutralization potential of alcoholic and aqueous extracts of *P. indica* on the %increase in survival rate in envenomed rats (LD_{50})

Treatment	N	% ↑ survival rate (1^{st} day)	% ↑ survival rate (5^{th} day)
Control	26	50	28.75
Standard (antivenom)	16	100	100
Aqueous extract (250mg/kg)	20	65	85.71
Aqueous extract (500mg/kg)	20	70	75.71
Alcoholic extract (250mg/kg)	20	80	80
Alcoholic extract (500mg/kg)	20	90	91.66

Table 6: Neutralization potential of aqueous and alcoholic extract of *P. indica* on *Viper russelli* venom-induced haemorrhagic and necrotic Activity

Treatment (N = 6)	MHD (in mm) Mean ± S.E.	MND (in mm) Mean ± S.E.
Control	8.733±0.257	4.186. ±079
Snake venom antiserum(Standard)	3.66±0.171	1.266±0.236
Aqueous extract (250mg/kg)	7.73±0.3414	4.43±0.143
Aqueous extract (500mg/kg)	6.35±0.3471	3.466±0.2
Alcoholic extract (250mg/kg)	5.58±0.230	4.15±0.099
Alcoholic extract (500mg/kg)	4.5±0.131	2.23±020

Table 7: Neutralization potential of aqueous and alcoholic extract of *P. indica* on mast cell degranulation

Treatment (N = 6)	(%Degranulated mast cells) Mean ± S.E	
	1st day	5th day
Control	77.89 ±3.09	78.96 ±4.94
Snake venom antiserum(Standard)	36.58±4.20	24.85±4.23
Aqueous extract (250mg/kg)	58.41±3.08	44.28±5.52
Aqueous extract (500mg/kg)	51.67±7.96	40.00±6.62
Alcoholic extract (250mg/kg)	55.75 ± 7.96	41.39 ± 5.04
Alcoholic extract (500mg/kg)	47.42 ± 8.27	36.94 ± 3.46

Table 8: *in Vivo* Antioxidant activity of the aqueous and alcoholic extract of *Pouzolzia indica* on TBARS levels of liver and kidney in *Viper russelli* envenomed rats

Treatment (N = 6)	TBARS (µmole/100g tissue) 1st day		TBARS (µmole/100g tissue) 5th day	
	Liver	*Kidney*	*Liver*	*Kidney*
Control	5.86 ± 0.09	4.56±0.09	6.39 ± 0.081	5.25±0.151
Snake venom antiserum	1.46±0.075	1.28±0.039	1.515±0.093	1.321±0.061
Aqueous extract (250mg/kg)	4.03±0.15	2.99±0.177	3.338±0.104	2.587±0.078
Aqueous extract (500mg/kg)	3.01±0.141	2.95±0.052	2.71±0.153	2.163±0.051
Alcoholic extract (250mg/kg)	3.98± 0.131	3.29±0.097	2.71± 0.118	2.221±0.129
Alcoholic extract (500mg/kg)	4.06±0.112	3.06±0.179	2.38±0.082	1.96±0.084

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