Research Article

Evaluation of Neutralization Potential of *Vipera russelli* Snake Venom By Extract of *Euphorbia hirta*

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ABSTRACT

The study was undertaken to investigate the evaluation of neutralization potential of *viperarusselli*snake venom by extract of *Euphorbia hirta*. The *Euphorbia hirta* whole plant was extracted using ethanol and tested for acute toxicity according to OECD-425 guidelines. The ethanolic extract of *Euphorbia hirta* at 100, 200, 400 mg/kg were evaluated for its efficacy to neutralize various actions of the venom like lethality, necrotizing activity, edema forming activity and hemorrhagic activity. Ethanolic plant extract when administered orally, effectively neutralized lethality induced by $2LD_{50}$ and $3LD_{50}$ of *viperarusselli*venomat dose levels 200 and 400mg/kg body weight(*in-vivo* neutralization). In *in-vitro* studies, plant extract at all dose levels, i.e. 100, 200 and 400mg/kg body weight effectively neutralized $2LD_{50}$ and $3LD_{50}$ of *viperarusselli*venom. It also significantly reduces viper venom induced hemorrhage, necrosis and edema at all dose levels in rats. Hence the present finding suggest that ethanolic extract of *Euphorbia hirta*plant possesses significant neutralizing capacity of snake *viperarusselli* venom which may be beneficial in the treatment of snake bite. Further study on isolation of active constituent from this plant extract is needed for development of new chemical antidote for snake envenomation.

Keywords: *Euphorbia hirta, viperarusselli* venom, Anti-venom, Hemorrhage, Necrosis, Edema.

INTRODUCTION

Snakebite is declared as a "Neglected Tropical Disease" by the World Health Organization. As a result, this may be considered as a matter of global health concern for the people in general and the rural communities of the developing countries in particular[1]. Nearly 200,000 persons fall prey to snakebite per year in India and approximately 35,000 – 50,000 lives are lost per year[2]. The common poisonous snakes found in India are Cobra (*Najanaja*), Krait (*BangarusCaeruleus*), Russell's viper (*Daboiarusselli*) and Saw Scaled Viper (*EchisCarinatus*)[3].

The venoms of cobra and krait are neurotoxic, that is, they affect the victim's central nervous system and cause heart failure. The venoms of Russell's viper and saw-scaled viper are histotoxicand hemorrhagic, therefore they provoke hemorrhagic manifestations that include epistaxis and cardiac manifestations such as myocarditis and cardiac failure[4]. Antiserum is the only therapeutic agent available for the snakebite[5]. But antiserum produces insufficient protection against snake bite, it fails to provide protective venom induced necrosis, hemorrhage, renal failure and its production is time consuming[5].

Over the years, many attempts have been made for the development of snake venom antagonists especially from plant sources. India has a rich tradition of the usage of medicinal plants. Many Indian medicinal plants are mentioned in literature, which are used to treat snakebite victims especially in rural areas[3].

*Euphorbia hirta*is a very popular herb commonly known as 'baridhudi' and belong to family *Euphorbiaceae*. Traditionally it is used to treat common diarrhoea, peptic ulcers, heartburn, vomiting, amoebic dysentery, asthma, bronchitis, hay fever, laryngeal spasms, emphysema, coughs, colds, kidney stones, menstrual problems, sterility, and venereal diseases. The whole plant is used to treat many disease and abnormalities in human[6].

Hence in the present investigation, an effort has been made to evaluate the anti-venom activity of ethanolic extract of whole plant

Euphorbia hirta against *Viperarusselli* venom by neutralizing capacity of lethality, hemorrhagic lesion, necrosis lesion and edema on experimental animals.

MATERIALS AND METHODS

Snake Venom

The lyophilized snake venom of *ViperaRusselli*was obtained from Hindustan Park, Kolkata, India and was preserved at 4^0C. Before use, the venom was dissolved in saline and required concentrations were prepared.

Snake Venom Antiserum

Lyophilized polyvalent snake venom antiserum (as reference serum) was obtained from Justice KS Hegde Charitable Hospital, Deralekatte, Mangalore.

Plant Material

The plant material was collected from Mangalore, Karnataka, India during May 2014 and was authenticated by Dr. Krishna Kumar, Associate Professor, Department of Applied Botany, Mangalore University.

Animals

Healthy adult Wistar albino rats, weighing about 180-220g and Swiss albino mice, weighing about 18-22g between 2 and 3 months of age obtained from KSHEMA, Deralakatte, Mangalore, were used for the study. The study was approved by the Institutional Ethics Committee for animal experimentation KSHEMA, Deralakatte, Mangalore. Rats and mice were housed individually in polypropylene cages, maintained under standard conditions (12 hlight and 12h dark cycle; 25^0C and 45-55% relative humidity). They had been given Standard pellet diet supplied by Hindustan Lever Co. Mumbai and water *ad libitum* throughout the course of the study.

Preparation of Extract

The fresh plant material of *Euphorbia hirta*(*Euphorbiaceae*) was washed and shade dried at room temperature. The air dried plant material was ground (1kg) and subjected for maceration. For extraction, the powdered plant material was soaked in ethanol and kept aside for 7 days with occasional stirring. After 7 days, the ethanolic layer was filtered. The solvent from the total extract was distilled off and the concentrate was evaporated on a water bath to a syrupy consistency and then evaporated to dryness[7].

Phytochemical Screening

The preliminary phytochemical studies were performed for testing the different chemical constituent present in ethanolic extract *Euphorbia hirta*[8,9].

Acute Toxicity Studies

Acute toxicity study was conducted to determine the median lethal dose (LD_{50}) of the ethanolic extract of plant *Euphorbia hirta*. The acute toxicity studied was carried out in female albino rats by “up and down” method (OECD guidelines 425). The animals were fasted overnight and extracts of the whole plant of *Euphorbia hirta*suspended in 0.6% CMC was administered starting at 2000 mg/kg. Then the animals were observed continuously for 3h for general behavioral, neurological, autonomic profiles and then every 30 min for next 3h and finally death after 24h[10].

Evaluation of LD_{50} of venom

The median lethal dose (LD_{50}) of *ViperaRusselli* venom was determined according to the method developed by Theakston and Reid 1983. The toxicity of *ViperaRusselli*venom was assessed by i.p administration of different concentrations of the venom dissolved in 0.2 ml of physiological saline to groups (n=6) of Swiss albino mice (18-22g). The LD_{50} was calculated with the confidence limit at 50% probability by the analysis of deaths occurring within 24h of the venom administration[11].

Neutralization of Lethality

The *in-vivo* neutralization potency of *Euphorbia hirta*plant extracts were assessed by i.p. administration of $2LD_{50}$ and $3LD_{50}$ dose of venom into different groups of mice immediately after the administration of various doses of the plant extract per oral (po)[11]. To assess *in-vitro* neutralization, various amounts of the plant extracts were mixed with $2LD_{50}$ and $3LD_{50}$ of the venom sample and incubated at 37^0C for 30 minutes and then injected i.p. in to the mice. 6 mice were used in each group. Control mice received same amount of venom without plant extracts. The standard reference group i.e. snake venom anti serum was administered after the administration of $2LD_{50}$ and $3LD_{50}$ dose of venom and the results were calculated by probit analysis[11].

Neutralization of Hemorrhagic Activity

The minimum hemorrhagic dose (MHD) of *Viperarusselli*venom was determined by the method described by Theakston and Reid, 1983. The minimum hemorrhagic dose is defined as the least amount of venom which

when injected intradermally (i.d.) in to rats results in a hemorrhagic lesion of 10mm diameter in 24h. The MHD of the venom was intradermally injected in to the shaved dorsal skin of the rats followed after 5 min by oral administration of different doses of the plant extract[11,12].

Neutralization of Necrotizing Activity

The minimum necrotizing dose (MND) of *ViperaRusselli*venom was determined by the method described by Theakston and Reid, 1983. The minimum necrotizing dose (MND) is defined as the least amount of venom (µg dry weight) which, when injected intradermally into rats, results in a necrotic lesion of 5 mm diameter 3 days later. The MND of venom was intradermally injected into the shaved dorsal skin of the rats followed after 5 min by oral administration of different dose of the plant extract[11].

Neutralization of Edema forming activity

The minimum edematic dose (MED) of venom/ carrageenan is defined as the least amount of venom/carrageenan which, when injected in to male albino rats**,** produced inflammation (edema) in the paw. To assess MED, Non fasted albino rats (180-220g) were treated with different dose of venom (in 0.1 ml) and were injected into sub-plantar area of the paw. Test group received MED of venom (sub-plantar) followed by different dose of plant extract per oral. As a control, only the venom was injected (sub-plantar). The edematogenic response was evaluated by the use of plethysmograph. Results were expressed as the percentage decrease in edema volume of the treated group compared to control[13].

Statistical Analysis

The lethal dose (LD_{50}) of the venom was expressed as µg/mice and was calculated by probit analysis. The other data's were expressed as Mean±SEM, analyzed by one way ANOVA followed by Dunnett's multiple comparison test[14].

RESULTS

Preliminary phytochemical Screening

The preliminary phytochemical screening of ethanolic extract of *Euphorbia hirta*revealed the presence of alkaloids, flavanoids, glycosids, steroids, tannins, saponnins, proteins and carbohydrates.

Acute Toxicity Studies

The ethanolic extract of the plant *Euphorbia hirta*was found to be safe upto 2000mg/kg body weight by oral route. After 24h animals were found well tolerated. There was no mortality and no signs of toxicity and extract were found to be safe.

Evaluation of LD_{50} of the Venom

The median lethal dose (LD_{50}) of *ViperaRusselli*venom was determined according to the method developed by Theakston and Reid 1983. The LD_{50} was calculated with the confidence limit at 50% probability by the analysis of deaths occurring within 24h of the venom administration. The LD_{50} of *Viperarusselli*venom was found to be 16 µg/20gmmice (i.p.).

***In-vivo* neutralization of Lethality**

The neutralization potency of *Euphorbia hirta*plant extracts by *in-vivo* method were assessed by i.p. administration of $2LD_{50}$ and $3LD_{50}$ dose of venom into different groups of mice (n=6) immediately after the administration of various doses of the plant extract per oral (po). The plant extract at doses 200 and 400mg/kg body weight were found to effectively neutralize the lethal activity of $2LD_{50}$ and $3LD_{50}$ of *Viperarusselli*venom(Table 1 & 2).

Table 1: Effect of *Euphorbia hirta*extract in mice administered with $2LD_{50}$ (32µg) of *Viperarusselli*venom (*in-vivo*).

Group	Dose of the Drug	Mortality (after 24h) [no. of death/no. of mice used]	% Survival after 24h	% Corrected	Probit
1	Control (only venom)	6/6	-	4.16	3.25
2	Venom + Std (Polyvalent anti-venom)	0/6	100	95.83	6.75
3	Venom + *Euphorbia hirta* 100mg/kg	3/6	50	50	5.00
4	Venom + *Euphorbia hirta* 200mg/kg	2/6	66.66	66.66	5.44
5	Venom + *Euphorbia hirta* 400mg/kg	2/6	66.66	66.66	5.44

Table 2: Effect of *Euphorbia hirta*extract in mice administered with $3LD_{50}$ (48µg) of *Viperarusselli*venom (*in-vivo*)

Group	Dose of the Drug	Mortality (after 24h) [no. of death/no. of mice used]	% Survival after 24h	% Corrected	Probit
1	Control (only venom)	6/6	-	4.16	3.25
2	Venom + Std (Polyvalent anti-venom)	0/6	100	95.83	6.75
3	Venom + *Euphorbia hirta* 100mg/kg	4/6	33.33	33.33	4.56
4	Venom + *Euphorbia hirta* 200mg/kg	4/6	33.33	33.33	4.56
5	Venom + *Euphorbia hirta* 400mg/kg	3/6	50	50	5.00

***In-vitro* neutralization of Lethality**

To assess *in-vitro* neutralization, various amounts of the plant extracts were mixed with $2LD_{50}$ and $3LD_{50}$ of the venom sample and incubated at 37^0C for 30 minutes and then injected i.p in to the mice. The plant extract at doses 200 and 400mg/kg body weight were found to effectively neutralize the lethal activity of $2LD_{50}$ and $3LD_{50}$ of *Viperarusselli* venom (Table 3 & 4).

Table 3: Effect of *Euphorbia hirta* extract in mice administered with $2LD_{50}$ (32µg) of *Viperarusselli*venom (*in-vitro*)

Group	Dose of the Drug	Mortality (after 24h) [no. of death/no. of mice used]	% Survival after 24h	% Corrected	Probit
1	Control (only venom)	6/6	-	4.16	3.25
2	Venom + Std (Polyvalent anti-venom)	0/6	100	95.83	6.75
3	Venom + *Euphorbia hirta* 100mg/kg	1/6	83.33	83.33	5.95
4	Venom + *Euphorbia hirta* 200mg/kg	0/6	100	95.83	6.75
5	Venom + *Euphorbia hirta* 400mg/kg	0/6	100	95.83	6.75

Table 4: Effect of *Euphorbia hirta* extract in mice administered with $3LD_{50}$ (48µg) of *Viperarusselli*venom (*in-vitro*)

Group	Dose of the Drug	Mortality (after 24h) [no. of death/no. of mice used]	% Survival after 24h	% Corrected	Probit
1	Control (only venom)	6/6	-	4.16	3.25
2	Venom + Std (Polyvalent anti-venom)	0/6	100	95.83	6.75
3	Venom + *Euphorbia hirta* 100mg/kg	2/6	66.66	66.66	5.44
4	Venom + *Euphorbia hirta* 200mg/kg	1/6	83.33	83.33	5.95
5	Venom + *Euphorbia hirta* 400mg/kg	0/6	100	95.83	6.75

Neutralization of Hemorrhagic Activity

The minimum hemorrhagic dose (MHD) of *ViperaRusselli*venom was determined by the method described by Theakston and Reid, 1983. The MHD of the venom was found to be 32µg/200g rat. The plant extract showed significant neutralization of hemorrhage at all the dose levels, when compared with the standard polyvalent anti-venom (Table 5, fig-1).

Table 5: Effect of ethanolic extract of *Euphorbia hirta*on the *Viperarusselli*venom induced hemorrhagic activity in rat

Dose of theDrug	Mean area of lesion ± S.E
Control (Only venom)	10.08±0.2007
Venom + Std (Polyvalent anti-venom)	9.583±0.2486
Venom + *Euphorbia hirta* 100mg/kg	4.167±0.1667
Venom + *Euphorbia hirta* 200mg/kg	3.25±0.2141
Venom + *Euphorbia hirta* 400mg/kg	1.583±0.2007

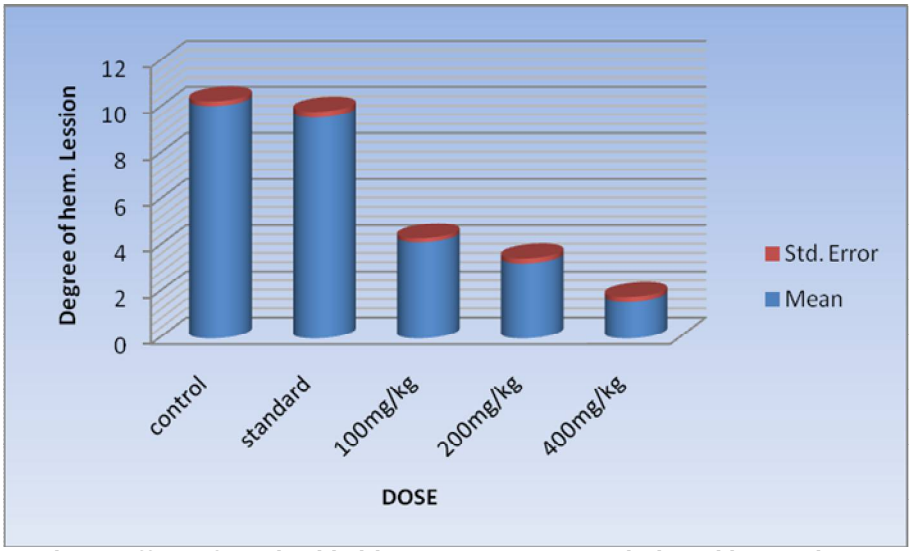

Fig. 1: Effect of *Euphorbia hirta*extract on venom induced hemorrhage

Neutralization of necrotizing activity

The minimum necrotizing dose (MND) of *ViperaRusselli venom* was determined by the method described by Theakston and Reid, 1983. The MND of the venom was found to be 40µg/200g rat. Plant extract showed significant activity at all the dose levels when compared with standard polyvalent anti-venom (Table 6, fig-2).

Table 6: Effect of ethanolic extract of *Euphorbia hirta*on the *Viperarusselli*venom induced necrotizing activity in rat

Dose of theDrug	Mean dia. of lesion ±S.E
Control (Only venom)	4.583±0.200
Venom + Std (Polyvalent anti-venom)	4.417±0.153
Venom + *Euphorbia hirta* 100mg/kg	2.667±0.166
Venom + *Euphorbia hirta* 200mg/kg	1.667±0.166
Venom + *Euphorbia hirta* 400mg/kg	0.750±0.250

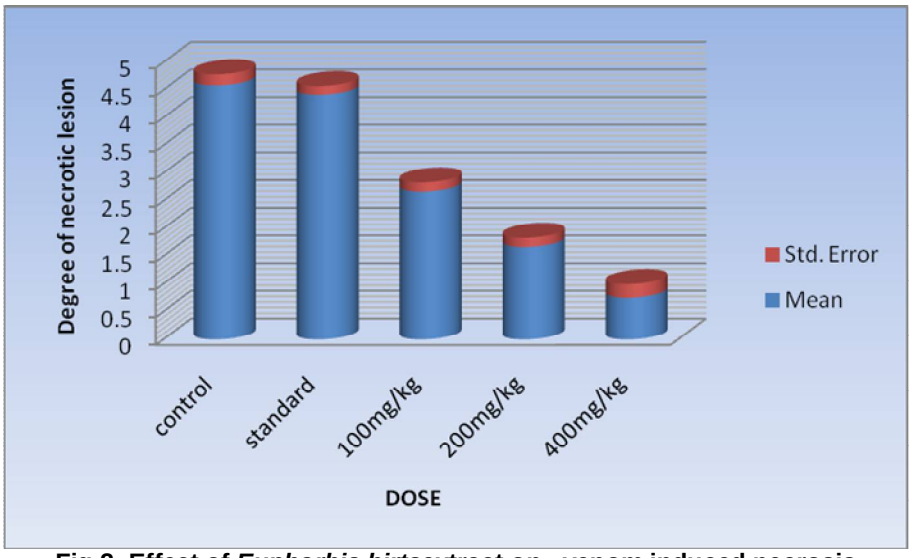

Fig 2: Effect of *Euphorbia hirta*extract on venom induced necrosis

Neutralization of Edema forming activity

The minimum edematic dose (MED) of venom/ carrageenan is defined as the least amount of venom/ carrageenan which, when injected in to male albino rats**,** produced inflammation (edema) in the paw. The minimum edematic dose of the venom was found to be 6µgin rat. Significant inflammation was seen after 1h of venom injection and maximum inflammation was seen at 180 min. Plant extracts at dose level 200mg/kg and 400mg/kg showed significant activity when compared with control.

Table 6: Effect of ethanolic extract of *Euphorbia hirta* on the *Viperarusselli*venom induced paw edema in rat

Dose of theDrug	Increase in edema volume		
	60min	120min	180min
Control (Only venom)	0.516±0.0307	0.633±0.0210	0.65±0.0223
Venom + Std (Polyvalent anti-venom)	0.3±0.0258	0.383±0.0307	0.35±0.0223
Venom + *Euphorbia hirta* 100mg/kg	0.45±0.0223	0.55±0.0258	0.516±0.0216
Venom + *Euphorbia hirta* 200mg/kg	0.4±0.0258	0.45±0.0223	0.416±0.0166
Venom + *Euphorbia hirta* 400mg/kg	0.266±0.0210	0.366±0.0333	0.3±0.0258

DISCUSSION

Snake envenomations cause different pathophysiological changes such as severe hemorrhage with haemostatic disturbances, bleeding and renal failure. In addition, pain, inflammation, edema and local wound necrosis are usually the main problems at the bite site[3].

The present study was undertaken to carry out the anti-venom activity of ethanolic extract of *Euphorbia hirta* whole plant belonging to family *Euphorbiaceae*. Preliminary phytochemical investigation revealed the presence of alkaloids, flavanoids, glycosids, steroids, tannins, saponins, proteins and carbohydrates. *Viperarusselli*venom is predominantly vasculo and haemotoxic, but able to produce neurotoxic effect also[17]. Hypotension is the important manifestation in all viper bites. Toxic symptoms include hemorrhage, renal failure, hypotension, local tissue necrosis, edema etc[3]. In the present study also, similar sequence of symptoms were observed after the administration of *Viperarusselli*venom.

Even though antiserum causes some side effects like hypersensitivity, anaphylactic reactions, it is the only therapeutic agent used for snake envenomations throughout the world. Several studies are going on to find out the suitable drug which can neutralize or antagonise the snake venom.

Different plant constituents such as alkaloids, acids, flavanoids, triterpinoids, tannins etc are responsible for the anti-snake venom activity[18].

Whole plant and roots of *Euphorbia hirta* have been widely used in folk medicine as an antidote for snake envenomations[19].

Three doses of the plant extract 100, 200 and 400 mg/kg body weight were selected based on the acute toxicity studies in rats.

*Viperarusselli*venom has an ability to cause local tissue damages such as necrosis and hemorrhage when injected intradermally. Hence the minimum necrotizing, minimum hemorrhagic dose and also minimum edematic dose estimation proves a reasonable test for assessing the anti-venom activity.

Ethanolic extract of whole plant of *Euphorbia hirta*at dose levels 100, 200 and 400 mg/kg body weight were used for the study.

LD_{50} of the venom was found to be 16µg/kg, MHD was 32µg in rats when injected intradermally after 24h, and MND was 40µg in rats when injected intradermally after three days and MED was found to be 6µg when injected by sub-plantar route in rats.

Ethanolic plant extract when administered orally, effectively neutralized lethality induced by $2LD_{50}$ and $3LD_{50}$ of *viperarusselli venom* at dose levels 200 and 400mg/kg body weight(*in-vivo* neutralization). In *in-vitro* studies, plant extract at all dose levels, i.e. 100, 200 and 400mg/kg body weight effectively neutralized $2LD_{50}$ and $3LD_{50}$ of *viperarusselli*venom.

*Viperarusselli*venom possesses the ability to cause local necrosis and hemorrhage when introduced intradermally. Hence the minimum necrotizing, minimum hemorrhagic dose estimation proves a reasonable test for assessing the anti-venom activity. The ethanolic extract of *Euphorbia hirta*whole plant significantly reduced viper venom induced hemorrhage and necrosis in rats.

The crisis of anti-venom supply especially in developing countries reflects a global loss of momentum in anti-venom research, development and financing. Failure of polyvalent anti-venom in neutralization of local tissue damage also forces the world to find newer alternative ways of treating snake bites. Our findings confirm the potent snake venom neutralization capacity of ethanolic extract of *Euphorbia hirta*.

Further study on isolation of active constituent from this plant extract and its anti snake venom activity could lead to development of new chemical antidote for snake envenomation.

CONCLUSION

The following conclusion can be drawn from the results obtained from present study. Orally administered ethanolic extract of *Euphorbia hirta*effectively neutralizes the lethality induced by $2LD_{50}$ and $3LD_{50}$ of *Viperarusselli*venom in rats. The extract is also able to reduce the venom induced necrosis and hemorrhage. It also has the potential to significantly reduce the venom induced edema.

Our findings confirm the potent snake venom neutralization capacity of ethanolic extract of *Euphorbia hirta.* Hence further study on isolation of active constituent from this plant extract and its anti snake venom activity is required.

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