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Antivenom activity of ethanolic extract of *Crescentia cujete* fruit

Shastry CS[1]*, Bhalodia Maulik M[1], Aswathanarayana BJ[1]

*Corresponding author:

Shastry CS

1 Department of Pharmacology,
NGSM Institute of Pharmaceutical Sciences,
Deralakatte, Mangalore - 575 018,
Karnataka, India

Abstract

In almost every part of the world, where venomous snakes occur, numerous plant species are used as folk medicine to treat snake bite. Ayurveda states usage of specific plants against specific snake bite. Hence the present study was carried out to evaluate the antivenom activity of ethanolic extracts of *Crescentia cujete* fruit in experimental animals.

The *Crescentia cujete* fruit was extracted using ethanol and tested for acute toxicity according to OECD 425 guidelines. The ethanolic extract of *Crescentia cujete* fruit at 100, 200 and 400 mg/kg were screened for the antivenom activity by *in-vivo* and *in-vitro* neutralizing capacity of lethality and neutralization of haemorrhagic lesion.

The fruit extract at dose levels of 200 and 400 mg/kg effectively inhibited *Vipera russelli* venom induced *in-vitro* lethality with 83.33% and 100% survival rate against $2LD_{50}$ respectively. The survival rate remains 50% and 83.33% with 200 and 400 mg/kg doses respectively against $3LD_{50}$. Whereas dose of 400 mg/kg significantly inhibit venom induced *in-vivo* lethality with 66% and 50% survival rate against $2LD_{50}$ and $3LD_{50}$ respectively. Haemorrhagic lesion was neutralizing at both dose levels with highly significant at 200 mg/kg dose.

Hence the present findings suggest that ethanolic extract of *Crescentia cujete* fruit possesses significant neutralizing capacity of snake *Vipera russelli* 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.

Key words: *Crescentia cujete* fruit; *Vipera russelli* venom; antivenom; lethality; haemorrhagic lesion.

Introduction

Snakebite envenoming constitutes a highly relevant public health issue on a global basis, although it has been systematically neglected by health authorities in many parts of the world. So snakebite is a global medical problem especially in the rural areas of the tropics with about 40,000 deaths each year. In India, more than 200,000 cases are reported and an estimated 35,000 to 50,000 people die of snakebite every year [1]. Approximately 330 species of snakes exist in India, of which about 70 species are venomous (40 land snakes and 30 sea snakes). The commonest Indian venomous snakes are, common krait (*Bungarus caeruleus*), common cobra (*Naja naja*), saw-scaled viper (*Echis carinatus*), and Russell's viper (*Vipera russelli*) [2]. Russell's viper is a nocturnal snake, frequently encountered by rural workers [3]. Antiserum is the only therapeutic agent so far available throughout the world for snakebite. But antiserum sometime does not provide enough protection against venom-induced haemorrhage, necrosis and often produces hypersensitive reactions. Also preparation of antiserum in animal is time consuming, expensive and requires ideal storage conditions [4]. Hence there is a search for a better medication over the available therapy and the world is looking for an alternative therapy for snake bite treatment. Till date no alternative measures are available, except the natural herbal remedies, which are showing promising results. The advantages of herbal compounds are, they are economic, easily available, stable at room temperature and could neutralize wide range of venom antigen. The herbal compounds could also effectively neutralize the snake venom in presence of anti-venom serum [5]. Over the years, many attempts have been made to develop an efficient snake venom antagonist especially from plant sources. India has a rich tradition of the usage of medicinal plants and many of Indian medicinal plants are mentioned in literatures, which are used to treat snakebite victims especially in the rural areas but lacking scientific validation. The roots, leaves, bark of many plants were mentioned in Ayurveda for the treatment of snake bite like, root extract of *Abrus precatorius* was used against krait bite and leaf paste of *Azadirachta indica* with rock salt was used against viper bites [5].

Crescentia cujete, commonly known as the Calabash tree belonging to family Bignoniaceae is a flowering plant that is native to Central and South America and is naturalized in India [6]. The fruit of *Crescentia cujete* is a part of the herbal mixtures reported in various traditional medicine for respiratory ailments, bronchitis, cough, colds, toothaches, headaches, as laxative, anti-inflammatory and febrifuge [7]. The plant has scientifically proved for cytotoxic and antibacterial activity [8,9].

Hence in the present investigation, an effort has been made to evaluate the antivenom activity of ethanolic extract of *Crescentia cujete* fruit against *Vipera russelli* venom by neutralizing capacity of lethality and haemorrhagic lesion on experimental animals.

Materials and methods

Chemicals

The lyophilized snake venom of *Vipera russelli* was procured from Calcutta Snake Park, Kolkata, India and lyophilized polyvalent snake venom antiserum (as reference serum) was obtained from Justice K S Hedge Charitable Hospital, Deralakatte, Mangalore, India which were preserved at 4 ^{c}ob Sodium chloride procured from High media, Bombay, India and all other chemicals were of analytical grade and used as received.

Plant material

The fruits of *Crescent curette* were collected from different places in Dashing Kannada district where it is grown as ornamental plant, during May 2011. It was authenticated by Dr. Newline J. Pinto, Head of Botany Department, St. Agnes College, and Mangalore. A voucher specimen (NGSMIPSM/Ph.cog/herb/15/2011) is retained in the department for further use. The fresh *Crescent curette* fruit pulp dried at 37°C in hot air oven for three days. The dried fruit pulp was ground and subjected for cold maceration with ethanol. The extract was concentrated to a syrupy consistency and then evaporated to dryness.

Preliminary phytochemicals screening

The preliminary phytochemicals studies were performed for testing different chemical constituents present in ethanol extract of *Crescent curette* [10].

Experimental animals:

Healthy adult Westar albino rats, weighing about 180-220g and Swiss albino mice, weighing about 18-22g between 2 and 3 months of age were used for the study. Animals were kept in the animal house of NGSM Institute of Pharmaceutical Sciences, Mangalore under controlled conditions of temperature (23±2°C), humidity (50±5%) and 12 hrs light-dark cycle. Animals were fed pellet diet (Venkateshwara enterprises, Bangalore) and water *ad libitum*. All the animals were acclimatized for seven days before the study. The experimental protocol was approved by institutional animal ethical committee (approval number: KSHEMA/AEC/35/2011)

Acute toxicity test

The acute toxicity study ethanolic extract of *Crescentia cujete* was carried out on rats by "up and down" method (OECD guidelines 425) [11].

Experimental design

For the evaluation of antivenom activity, three dose levels 100 mg/kg, 200 mg/kg and 400 mg/kg were selected.

Evaluation of LD_{50} of the venom

The median lethal dose (LD_{50}) of *Vipera Russelli* venom was determined according to the method developed by Theakston and Reid, 1983. The toxicity of *Vipera russelli* venom was assessed by intra peritoneal (i.p.) administration of different concentrations of the venom dissolved in physiological saline to groups 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 24 hrs of the venom administration [12].

Neutralization of lethality

The study comprises 5 groups of 6 animals each as follows.
Control group receives *Vipera russelli* venom only
Standard group receives *Vipera russelli* venom and snake venom antiserum
Ethanolic extract of *Crescentia cujete* (100 mg/kg) with *Vipera russelli* venom
Ethanolic extract of *Crescentia cujete* (200 mg/kg) with *Vipera russelli* venom
Ethanolic extract of *Crescentia cujete* (400 mg/kg) with *Vipera russelli* venom

In-vivo neutralization

The *in-vivo* neutralization potency of *Crescentia cujete* 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 oral administration of various doses of the plant extract. Results were analyzed by probit analysis of deaths occurring within 24 hrs of the various treatments.

In-vitro neutralization

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° C for 30 minutes and then injected i.p. in to the mice. Results were analyzed by probit analysis of deaths occurring within 24 hrs of the various treatments [12].

Neutralization of haemorrhagic activity

The minimum haemorrhagic dose (MHD) of *Vipera Russelli* venom was determined by the method described by Kondo et al

in rats. The MHD is defined as the least amount of venom which when injected intradermally (i.d.) in to rats results in a haemorrhagic lesion of 10 mm diameter in 24 hrs.
The MHD of the venom was i.d. injected in to the shaved dorsal skin of the rats followed after 30 min by oral administration of different doses of the plant extract [12,13].
The study comprises 5 groups with 6 animals each as follows.
Control group receives MHD of *Vipera Russelli* venom only
Standard group receives MHD of *Vipera Russelli* venom and snake venom antiserum
Ethanolic extract of *Crescentia cujete* (100 mg/kg) with MHD of *Vipera Russelli* venom
Ethanolic extract of *Crescentia cujete* (200 mg/kg) with MHD of *Vipera Russelli* venom
Ethanolic extract of *Crescentia cujete* (400 mg/kg) with MHD of *Vipera Russelli* venom
The dorsal skin was removed after 24 hours of intradermal injection of *Vipera Russelli* venom and the diameter of haemorrhagic lesion was measured.

Statistical analysis

The lethal dose (LD_{50}) of the venom was expressed as µg/mouse and was calculated by probit analysis [14]. The other data were expressed as mean ± standard error of the mean (S.E.M.) of 6 animals per group. Parametric one way analysis of variance (ANOVA) followed by Dunnett's test. Statistical analysis was performed using Graph pad prism 5.0. The minimal level of significance was identified at $P < 0.05$.

Results

Phytochemical screening

The preliminary phytochemical screening of the ethanolic extract of *Crescentia cujete* (CC) fruit revealed the presence of alkaloids, flavanoids, glycosides, resins, steroids and tannins. (Table 1)

Table 1: Qualitative analysis of ethanolic extract of CC

S. No	Tests	Inference
1	Alkaloids a) Dragendorff's test b) Hager's test c) Wagner's test d) Mayer's test	 + ve + ve + ve - ve
2	Carbohydrates a) Anthrone test b) Benedict's test c) Fehling's test d) Molisch's test	 - ve - ve - ve - ve
3	Flavanoids a) Shinoda's test	 + ve
4	Glycosides a) Molisch's test	 + ve
5	Triterpenoids a) Liebermann – Burchard test	 - ve
6	Resins	+ ve
7	Saponins	- ve
8	Steroids a)Liebermann -Burchard's test b)Salkowski reaction	 + ve + ve
9	Tannins	+ ve

+ve = Positive/present, -ve = Negative/absent

Table 2: Effect of VR venom on mice to determine the median lethal dose (LD_{50}) of the VR venom.

Group	Dose of venom (µg/kg)	No. of deaths / no. of mice used	% Death	% Corrected*	Probit
1	200	0/6	-	04.16	3.25
2	400	1/6	16.66	16.66	4.05
3	600	3/6	50.00	50.00	5.00
4	800	4/6	66.66	66.66	5.44
5	1000	6/6	100.00	95.83	6.75

No. = Number.

*corrected formula for the 0% dead: 100(0.25/n)For the 100% dead: 100[(n-0.25)/n] Where n is the number of animals in the group

Table 3: *In-vivo* neutralization effect of CC fruit extract in mice administered with $2LD_{50}$ (1200 µg/kg) of VR venom.

Sl no.	Group	VR venom 2 LD_{50}			
		Mortality	% survival after 24 hrs	Corrected %	Probit
1	Control (VR venom)	6/6	-	4.16	3.25
2	VR venom + Standard antivenom.	0/6	100	95.83	6.75
3	VR venom + ethanolic extract of CC-100mg/kg	4/6	33.33	33.33	4.56
4	VR venom + ethanolic extract of CC- 200mg/kg	3/6	50.00	50.00	5.0
5	VR venom + ethanolic extract of CC - 400mg/kg	2/6	66.66	66.66	5.44

Mortality = no. of death/no. of mice used

Table 4: *In-vivo* neutralization effect of CC fruit extract in mice administered with $3LD_{50}$ (1800 µg/kg) of VR venom.

Sl no.	Group	VR venom 3 LD_{50}			
		Mortality	% survival after 24hrs	Corrected %	Probit
1	Control (VR venom)	6/6	-	4.16	3.25
2	VR venom + Standard antivenom.	0/6	100	95.83	6.75
3	VR venom + ethanolic extract of CC 100mg/kg	5/6	16.66	16.66	4.05
4	VR venom + ethanolic extract of CC- 200mg/kg	5/6	16.66	16.66	4.05
5	VR venom + ethanolic extract of CC - 400mg/kg	3/6	50.00	50.00	5.00

Mortality = no. of death/no. of mice used

Acute toxicity study

The ethanolic extract of CC fruit was found to be safe up to 2000mg/kg body weight by oral route. After 24 hrs animals were found well tolerated. There was no mortality and signs of toxicity after 24 hrs. Three dose levels i.e. 100mg/kg, 200mg/kg and 400mg/kg body weight were selected for the present study.

Evaluation of LD_{50} of the *Vipera russelli* venom

The LD_{50} was calculated with the confidence limit at 50% probability by the analysis of deaths occurring within 24 hrs of the *Vipera Russelli* (VR) venom administration and was found to be 600 µg/kg mice (i.p.). (Table 2)

Neutralization of lethality:

Effect of ethanolic extract of CC fruit on in-vivo neutralization of lethality

The ethanolic extract of CC at dose of 400 mg/kg significantly found to neutralize with 66% survival rate upon the lethal activity of $2LD_{50}$ (1200 µg/kg) and with 50% survival rate with lethal activity of $3LD_{50}$ (1800 µg/kg) VR venom. (Table 3 and 4)

Effect of ethanolic extract of CC fruit on in-vitro neutralization of lethality

The ethanolic extract of CC at doses 200 mg/kg has significant *in-vitro* neutralization activity with 83.33% and 50% of survival rate with $2LD_{50}$ and $3LD_{50}$ lethal activity respectively. Whereas the higher dose of CC at 400 mg/kg were found to be highly effective *in-vitro* neutralization activity with 100% and 83.33% survival rate with $2LD_{50}$ and $3LD_{50}$ respectively which is comparable with 100% survival rate of standard antivenom group with both$2LD_{50}$ and $3LD_{50}$ lethal activity . (Table 5 and 6)

Table 5: *In-vitro* neutralization effect of CC fruit extract in mice administered with $2LD_{50}$ (1200 µg/kg) of VR venom.

Sl no.	Group	VR venom 2 LD_{50}			
		Mortality	% survival after 24 hrs	Corrected %	Probit
1	Control (VR venom)	6/6	-	4.16	3.25
2	VR venom + Standard antivenom.	0/6	100	95.83	6.75
3	VR venom + ethanolic extract of CC 100mg/kg	3/6	50.00	50.00	5.0
4	VR venom + ethanolic extract of CC- 200mg/kg	1/6	83.33	83.33	5.97
5	VR venom + ethanolic extract of CC - 400mg/kg	0/6	100	95.83	6.75

Mortality = no. of death/no. of mice used

Table 6: *In-vitro* neutralization effect of CC fruit extract in mice administered with $3LD_{50}$ (1800 µg/kg) of VR venom.

Sl no.	Group	VR venom 3 LD_{50}			
		Mortality	% survival after 24 hrs	Corrected %	Probit
1	Control (VR venom)	6/6	-	4.16	3.25
2	VR venom + Standard antivenom.	0/6	100	95.83	6.75
3	VR venom + ethanolic extract of CC 100mg/kg	4/6	33.33	33.33	4.56
4	VR venom + ethanolic extract of CC- 200mg/kg	3/6	50.00	50.00	5.0
5	VR venom + ethanolic extract of CC - 400mg/kg	1/6	83.33	83.33	5.97

Mortality = no. of death/no. of mice used

Neutralization of haemorrhagic activity

Effect of ethanolic extract of CC fruit on neutralization of haemorrhagic activity.

The minimum haemorrhagic dose (MHD) of VR venom was found to be 24 µg/kg of body weight. The ethanolic extract of CC at 200 mg/kg showed highly significant (P<0.01) neutralization of haemorrhage than the standard antivenom group when compared with the control group and also 100 mg/kg and 400 mg/kg showed significant (P<0.05) neutralization of haemorrhage. (Table 7 and figure 1)

Table 7: Effect of ethanolic extract of CC fruit on neutralization of haemorrhagic activity

Group	Mean dia. of lesion ± S.E.M
Control (MHD of VR venom)	10.08±0.1537
MHD of VR venom + Standard antivenom.	9.583±0.1537
MHD of VR venom + ethanolic extract of CC 100mg/kg	9.250±0.2141*
MHD of VR venom + ethanolic extract of CC- 200mg/kg	4.000±0.1291**
MHD of VR venom + ethanolic extract of CC - 400mg/kg	9.167±0.2108*

The Values are expressed as Mean ± SEM, n=6 rats in one group. *P<0.05 significant, **P< 0.01 highly significant, when compared with control group.

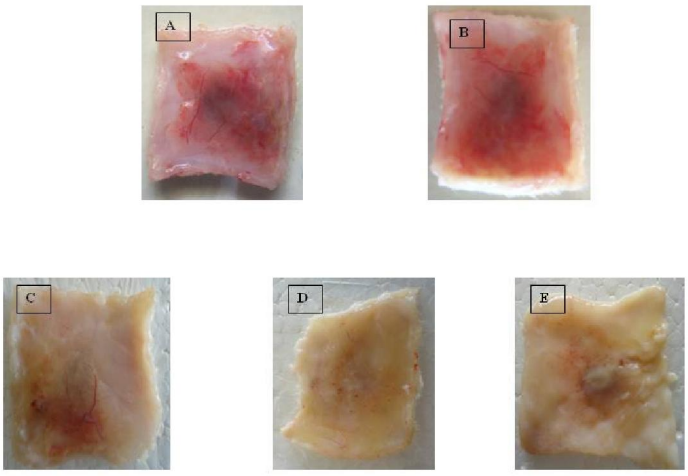

Figure 1: Effect of CC fruit extract on VR venom induced haemorrhagic activity in rat.
A-Control group showing maximum haemorrhagic lesion, B-Standard anti venom group shows no significant decrease in haemorrhagic lesion, C-CC 100 mg/kg slight significant decrease in haemorrhagic lesion, D-CC 200 mg/kg having highly significant reduction in haemorrhagic lesion, E-CC400 mg/kg slight significant decrease in haemorrhagic lesion compared to control group.

Discussion

Snake envenomations cause different pathophysiological changes such as inflammation, haemorrhage, necrosis, edema, alterations in blood coagulation system, and ultimately leading to death. *Vipera russelli* venom is predominantly vasculo and haemotoxic, but it also able to produce neurotoxic effects. The main toxic symptoms include haemorrhage, renal failure, hypotension, local tissue necrosis, edema etc. Hypotension is the important manifestation in all viper bites and it has been responsible for 38% of deaths in *Russell viper* envenomations [4]. In the present study similar sequence of symptoms were observed after the administration of *Vipera russelli* venom.

According to WHO, the anti snake venom activity possessing compound should be tested regarding its capacity to neutralize the in-vivo biological effects such as lethality, haemorhage, necrosis, edema etc [12]. The crisis of snake venom antivenom supply especially in developing countries reflects a global loss of momentum in anti venom research, development and financing. Failure of polyvalent antivenom in neutralization of local tissue damage also forces the world to find newer alternative ways of treating snake bites. Hence in the present study the ability of ethanolic extract of *Crescentia cujete* for the neutralizing capacity of lethality (*in-vivo* and *in-vitro*) and haemorrhagic lesion was studied.

Several studies have been carried out to find the suitable drug which can neutralize or antagonize the snake venom. Different plant constituents such as alkaloids, acids, flavanoids, triterpinoids, tannins etc are responsible for the anti-snake venom activity. *Crescentia cujete*, commonly known as Calabash tree belongs to family Bignoneaceae. Preliminary phytochemical screening revealed the presence of alkaloids, flavanoids, glycosides, resins, steroids and tannins in the ethanolic extract of *Crescentia cujete* fruit.

From the present study the ethanolic extract of *Crescentia cujete* fruit neutralized lethality induced by $2LD_{50}$ and $3LD_{50}$ of *Vipera russelli* venom at dose levels 400 mg/kg body weight (*in-vivo* neutralization) and in *in-vitro* studies at dose levels of 200 mg/kg and 400mg/kg. Hemorrhage produced by venom in rats was significantly inhibited by dose level of 200 mg/kg shows better anti-venom activity. Hence the anti-snake venom activity may be due to the presence of flavonoids, tannins and alkaloids in the extract.

Conclusion

Our findings confirm the moderate snake venom neutralization capacity of ethanolic extract of *Crescentia cujete* fruit. Further study on isolation of active constituent from this plant extract is needed for development of new chemical antidote for snake envenomation.

Authors' contributions

BM carried out the study. SC participated in the design of the study and reviews the manuscript. AB performed the statistical analysis and helped to draft the manuscript.

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Declarations: Conflict of interest

The author(s) declare(s) that they have no conflicts of interest to disclose.

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