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Inhibition of pharmacological and toxic effects of *Echis carinatus* venom by *Tabernaemontana alternifolia* root extract

Vineetha M S [1], Bhavya J[1] and Sunil S More[2*]

[1]Department of Biochemistry, Centre for Post Graduate Studies, Jain University, Bangalore -560011, India
[2]School of Basic and Applied Sciences, Dayananda Sagar University, Bangalore-560078, India

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The aim of the present study is to validate the anti-venom potential of traditionally used plant *Tabernaemontana alternifolia* root extract against *Echiscarinatus* venom by *in vitro* and *in vivo* studies. The methanol extract of the plant was evaluated for venom-induced pharmacological activities. Lethal toxicity determination, haemorrhagic studies and its neutralization were studied on chick embryo and mice model. Oedema and myotoxic neutralization studies were carried out on mice model. At venom:extract 1:60 (w/w) concentrations delayed the clotting time from 12.11 to 100.66 s and at 1:40 (w/w) concentration inhibited the fibrinogen degradation. Further extract significantly inhibited direct and indirect haemolytic activities. The effective dose of extract for 2 LD_{50} of the venom was 0.98 mg/egg. The venom: extract at 1:20(w/w) ratio effectively antagonized venom-induced lethal toxicity. Reference haemorrhagic dose was found to be 2 µg in chick embryo and 10.00 µg was minimum haemorrhagic dose in mice model. Extract completely eliminated the haemorrhage on mice and chick embryo model. Venom-induced oedema ratio of 154% was reduced to 128% and LDH activity from 4520 ± 6.36 to 1685.33 U/L. *T. alternifolia* possesses highly promising anti-snake venom activity, thus study provided scientific evidence for its traditional use against snakebites.

Keywords: Chick embryo model, *Echiscarinatus*, Haemorrhage, Procoagulant, *Tabernaemontana alternifolia*.

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Introduction

Envenomation by the snake is a major problem especially in South Asia, North Africa, Latin America, Middle East region of the world[1]. In 2009, the World Health Organization recognised snake bite as a "Neglected tropical disease"[2]. Snakebite causes both systemic (fatal) and local toxicities. Viper bites predominantly result in the local toxicity, characterized by continued tissue destruction. Anti-venom fails to neutralize viper venom-induced local tissue destructions. *Echis carinatus*, a member of "Big four" snake species of India, belongs to pitless viper family, primarily its bite disrupts the haemostatic system. *E. carinatus* bites kill more people compare to any other species in Northern Africa and Asia[3]. *E. carinatus* venom induces neutrophil extracellular trap (NET) formation[4]. The NETs entrap the venom toxin at the injection site, promoting tissue destruction[4]. Venomous bite of the snake is primarily responsible for bleeding associated with haemorrhage, pro-coagulant, bleeding effects and fibrinogenolytic effects. *E.carinatus* venom is pro-coagulant in nature and mechanism of venom-induced pro-coagulant effect due to activation of prothrombin[5 6].

The only available medical treatment for snakebite is the immediate administration of anti-serum to victims. Anti-serum will bind to the toxin and neutralize the toxic effects, which prevent further damages happening by the venom, but it's not effective against the damages already caused by the venom[7]. Polyvalent anti-serum available in India are raised against the Indian big four species of snakes. Antiserum is not effective against the local effects induced by the venom. It is associated with risk after administration such as early anaphylactic reaction, late anaphylactic reaction and pyrogenic reaction which needs further medical attention**.** Further, it is not easily accessible and the cost of anti-serum is high for the rural people. Considering the drawbacks associated with existing anti-serum therapy, systematic validation of plants for venom toxicity is justified.

WHO encourages on documenting locally available plants for the treatment of diverse health problems. If

*Correspondent author
Email: sunilacr@yahoo.co.in
Tel.: +919481787729

these locally available plants are scientifically evaluated and disseminated properly regarding the efficacy of the herbal treatment, it will improve the health condition due to snake bites[8]. The plant possesses active phytochemicals which have the antagonizing potential against the toxic enzymes of snake venom[9, 10]. In rural places snake bite victim's approaches the traditional healers for the snake bite treatments and it is mostly accepted by the rural people from many years. In Bangladesh only 10% of snake bite patients take the treatment in the hospital, the rest of them depended on herbal treatments. Plants like *Mimosa pudica, Vitex negundo, Emblica officinalis, Andrographis paniculata, Aristolochia indica, Vitis vinifera, Ophiorrhiza mungos* and many more were reported for its anti-venom activity[11-16].

Traditional healers have acquired outstanding knowledge about locally available plants for the treatment of various ailments. Plants are a well-known antidote for snake bites from centuries, knowledge of this is being used and was conserved among tribal communities. Several ethnobotanical studies were conducted which indicate various plants have been used in particular geographical location. *T. alternifolia* one of the plants extensively used in the herbal preparations to treat the snakebites in the Western Ghats region of Karnataka. Brazilian traditional medicine system uses an aqueous decoction of plants from *Tabernaemontana* genus for snake bite treatment. Anti-venom potential of *Tabernaemontana catharinensis* was reported for South American Rattlesnake venom-induced lethal toxicity and myotoxic effects[17]. Earlier we have reported *in vitro* neutralization of enzyme activities of *E. Carinatus* and *Naja naja* venom using *Tabernaemontana alternifolia* root extracts[18]. The methanol extract was promising compared to other extracts like ethyl acetate, acetone, ethanol and water extracts. The present study aimed to examine the effectiveness of methanol root extract against the *E. carinatus* venom-induced pharmacological and toxic effects using *in vitro* and *in vivo* models.

Material and Methods

Chemicals and venom

N,N,N'N'-Tetramethylethylenediamine(TEMED), acrylamide, tris base, fibrinogen and β-mercaptoethanol were procured from HiMedialaboratories (Mumbai, India). N-N'-Methylene-bisacrylamide, brilliant blue R, sodium dodecyl sulfate were purchased from Sigma. Calcium chloride and Folin-Ciocalteu reagent were from Qualigens fine chemicals (Mumbai, India). All the other chemicals used were of analytical grade.

The lyophilized *E. carinatus* venom was procured from Irula Snake Catcher's Co-operative Society, Kancheepuram, Chennai, Tamil Nadu, India. Venom was stored at 4 °C.

Extract preparation

T. alternifolia root was collected in the months of April 2015 from Kollur, Kundapura Taluk, Udupi District, Karnataka. The plant was collected from traditional healer for the study purpose. It was identified, authenticated (RRCBI-MUS/03) and deposited at National Ayurveda Dietetics Research Institute, Bangalore, Karnataka. The root powder was extracted using Soxhlet extractor as described previously[18]. Prior to use, plant extract was dissolved in saline and centrifuged at 2000 rpm for 10 min and the supernatant was used for further studies.

In vitro (Chick embryo model) and *in vivo* (Mice) models

Six-day-old eggs were procured from the Department of Poultry, Karnataka Veterinary College, Hebbal, Bangalore.

Swiss albino male mice weighing 25-30 g were obtained from Sri Raghavendra Enterprises, Bangalore (Registration number of the breeder 841/b/04/CPCSEA). Six mice were housed per polyvinyl cage and maintained at standard laboratory conditions of 23-25 °C, 12 h light and 12 h dark cycle, water and standard pellet diet provided ad libitum. One week prior to the experiment, animals were acclimatized to the laboratory condition. Animal care, handling and disposal were carried out according to the Institutional Animal Ethics Committee (IAEC). The ethical committee approval number is IAEC/NCP/92/2015.

Neutralization of procoagulant activity

Recalcification time of the human blood was determined according to the method described by Condrea *et al*[19]. Human citrated plasma of 300 µL was incubated with 30 µL Tris-HCl buffer (pH 7.6) at 37 °C for 15 minutes. To this reaction mixture, *E. carinatus* venom was added and clot formation was initiated by adding 30 µL of 0.25 M $CaCl_2$. Control tube consisted of human plasma with Tris-HCl buffer and $CaCl_2$. Coagulation time was expressed as a mean of coagulation time in the presence and absence of plant extract. Minimum coagulant dose (MCD) was defined as the venom concentration which induces clotting of plasma within 60 seconds. For inhibition

studies venom was pre-incubated with different concentration of *T. alternifolia* methanol extract. The concentration of anti-venom /plant extracts needed to increase the clotting time by two times when compared to the plasma clotting time (incubated with MCD of venom alone).

Neutralization of fibrinogenolytic activity

Fibrinogenolytic activity induced by the *E. carinatus* venom was determined by Ouyang and Teng method[20]. The reaction mixture consisted of 50 µg bovine fibrinogen in 10 mM Tris-HCl buffer pH 7.4 was incubated with the venom of different concentration for 1 h at 37 °C. The reaction was terminated by adding 20 µL of denaturing buffer containing 1 M urea, 4% SDS and 4% *β*-mercaptoethanol. The hydrolysed product was analysed with 12% SDS and the protein pattern was visualized by staining with Coomassie brilliant blue[21]. For neutralization study, methanol extracts of *T. alternifolia* were pre-incubated with *E. carinatus* venom.

***In vitro* HRBC lysis by *E. carinatus* venom (Direct haemolytic assay) and its stabilization Properties of plant extract**

Inhibition of *E. carinatus* venom-induced haemolysis by plant extract was measured *in vitro* by inhibition of human red blood corpuscles (HRBC) lysis. Hypo saline-induced haemolysis was used generally for studying the antioxidant properties of natural inhibitors was modified in present by venom-induced haemolysis[22]. The collected blood was washed thrice with saline and centrifuged at 2000 rpm for 15 min. The preparation of HRBC cell suspension was carried out according to the method given by Murugesh *et al*[23]. *E. carinatus* venom of 100 µg/mL was dissolved in physiological saline. The HRBC with 0.1 M phosphate buffer saline of pH 8.0 served as control. The reaction mixture contains HRBC, phosphate buffer and venom (100 µg in 1 mL). For inhibition study, venom was pre-incubated with *T. alternifolia* methanol extract of different concentration. The absorbance of the supernatant was measured at 540 nm using a spectrophotometer. The inhibition of per cent of haemolysis was calculated by the following equation,

$$\text{Inhibition of \% of haemolysis} = \text{Ac-At/At} \times 100$$

Where, A_c - Absorbance of control, A_t - Absorbance of test (plant extract) with venom solution

Indirect haemolysis assay (Phospholipase A_2 activity)

Phospholipase A_2(PLA_2) activity was measured by haemolysis of RBC on agarose-erythrocyte-egg yolk gel[24]. Human erythrocytes were washed several times with phosphate buffer saline, centrifuged and sediments were removed. The venom was loaded into 1.5 mm wells in agarose (0.8% in phosphate buffer saline) plate containing 1.2% of human erythrocyte and 1.2% egg yolk as a source of lecithin. The plates were incubated overnight at 37 °C and haemolytic halos were measured. Minimum haemolytic halo (MHH) is defined as venom concentration at which it produced a haemolytic halo of 10 mm. Inhibition studies were carried out by pre-incubating the *T. alternifolia* methanol extract of different concentration with MHH dose of venom. Saline and plant extract served as control.

Acute toxicity determination using chick embryo model

Acute toxicity of the *T. alternifolia* methanol extract was determined using chick embryo model[25,26]. Six days old eggs were surface sterilized with alcohol and a small hole was made by avoiding vasculature to transfer the plant solution. Methanol crude extract of 20 µg/mL and 200 µg/mL was dissolved in a fixed volume (0.2 mL) of saline and was transferred into the hole which was made in the eggs (n = 6) using an insulin syringe. The control group of eggs (n = 6) were injected with saline. The holes were sealed by using parafilm to avoid the contamination and desiccation of the contents inside. Eggs were incubated for 24 h in swinging incubator at 37 °C and 65% relative humidity for embryogenesis. Post 24 h of incubation, eggs were observed for survivability by using the candling technique. The embryos were considered dead if they were motionless and had no distinguishable vasculature. The accuracy of candling was confirmed by opening eggs at the apex and examining the embryos.

Acute oral toxicity determination using mice model

Acute oral toxicity of *T. alternifolia* methanol extract was performed according to the organisation for economic co-operation and development (OECD) guidelines 423 in the mice model. Plant extract of 2000 mg (dissolved in saline) per kg body weight was administrated to healthy mice (n = 5) by oral gavages. The mice were observed for mortality, behavioural changes, toxicity at least once a day for 14 days. The weight of the mice was recorded on the day of administration, on the 7th day and finally on the 14th day of the oral toxicity study. The control group of mice were injected with saline.

Determination of LD_{50} and neutralization of LD_{50} in chick embryo model

The lethal toxicity of *E. carinatus* venom was determined by using chick embryo model[25]. Modification to Sells *et al*. method was contents of the egg were not transferred out of the egg instead test sample was transferred inside the eggs at the apex without disturbing egg contents[26]. Six days old eggs were surface sterilized with alcohol and a small hole was made by avoiding the vasculature at the apex. The venom of different concentration in a constant volume of saline was transferred to egg using an insulin syringe. Whereas control group of the eggs (n = 6) were injected with saline. The holes were sealed by using wax to avoid the contamination and desiccation of the egg contents. Eggs were incubated for 24 h in swinging incubator at 37°C and 65% relative humidity for embryogenesis. After 24 h of incubation eggs were observed for survivability by using the candling technique. Eggs with no distinguishable vasculature were considered as dead. The accuracy of fast candling technique was further confirmed by the opening the eggs at the apex and examining the embryos. LD_{50} was calculated according to Meier and Theakston method[27].

For neutralization, 2 LD_{50} of the *E. carinatus* venom was taken as challenging dose. Neutralization of lethal toxicity was carried out by pre-incubating the venom with *T. alternifolia* methanol extract of different concentrations at 37°C. Challenging doses of the venom and pre-incubated venom with plant extracts were transferred into eggs (n = 6) using the insulin syringe. After 24 h of incubation, eggs were observed for survivability by using candling technique and examining the embryos. ED_{50} is defined as, concentration of anti-venom/plant extract needed to protect 50% of the embryos.

Lethal toxicity (LD_{50}) determination and LD_{50} neutralization in mice model

The median lethal dose of *E. carinatus* was determined according to the method developed by Theakston and Reid[28]. Deaths were recorded at 24 h and lethal toxicity calculation was done according to the Meier and Theakston method[27].

For inhibition study 3 LD_{50} of *E. carinatus* venom was selected as challenging dose. Neutralization of lethal toxicity was carried out by two different protocols. 1. Pre-incubation of venom with plant extracts 2. Separate injection of venom and plant extracts. For both the protocols *T. alternifolia* methanol extract of two different concentrations were used (1:10, 1:20 w/w). The first group of mice (n = 5) were administered with 3 LD_{50} venoms. For pre-incubation protocols challenging dose of venom was pre-incubated with methanol extract of two different concentrations which were administered to group two and three (n = 5) of mice. Separate injection protocol for two different concentrations of plant extract was injected into the same spot exactly after 5 min after venom injection into group 4 and 5.

Neutralization of haemorrhagic activity in chick embryo model

The haemorrhagic activity of the venom was determined by using the chick embryo model with slight modification[29]. Six days old eggs were used to study the haemorrhage activities. Modification made to Sells *et al.* was that the contents were not transferred out of shells but an opening was made at the apex of the egg. Previously prepared discs (2 mm diameter) were put on one of the vitelline veins of the egg embryo followed by re-incubation of eggs for 3 hours. Different concentrations of venom in a range of 1 to 5 µg/1.5 µL of phosphate buffer saline (PBS) were applied to the discs. Venom concentration which created a haemorrhagic corona of 2 mm was regarded as the reference haemorrhagic dose (RHD). A transparent ruler was used to measure the corona. Different concentrations of *T. alternifolia* methanol extract were incubated with RHD of venom at 37 °C for 30 minutes before applying to the discs. Saline served as control and plant extract of higher concentration was also analysed for haemorrhagic activity. The minimum concentration of antidote required (plant extract) to eradicate haemorrhage was considered as the minimum effective neutralizing dose (MEND).

Neutralization of haemorrhagic activity in mice model

Mice were intradermally injected with 50 µL saline containing venom of *E. carinatus*. Minimum haemorrhagic dose (MHD) is defined as the amount of venom required to produce a haemorrhagic halo of 10 mm. For neutralization of haemorrhagic activity, MHD of venom was pre-incubated with different concentration of *T. alternifolia* methanol extract for 30 minutes at 37°C and was injected to mice by intradermal route (n = 5). Reduction in haemorrhagic halo was measured[30]. After 3 hours, mice were anaesthetized and dorsal surface of the skin was removed and observed for haemorrhage.

Neutralization of Edema Inducing Activity

Neutralization of oedema by plant extract was studied using mice model[31]. Mice (n = 5) were

injected with 5 µg of venom in 20 µL of saline into right and left footpads, 20 µL of saline served as control. After 1 h, mice were anaesthetized, footpad was cut at the ankle joint and increase in weight due to oedema was measured. For the neutralization studies, 5 µg of venom was incubated with different concentration of methanol extract of *T. alternifolia*. Oedema ratio is equal to the weight of the edematous leg×100/weight of the control leg.

Neutralization of myotoxic activity

The myotoxic activity was determined by measuring the elevation of cytoplasmic marker enzyme lactate dehydrogenase (LDH) in the serum[32]. Group one mice (n = 5) and group two (n = 5) were injected with saline and half the LD_{50} of venom in 50 µL saline by intramuscular route. Group three (n = 5) and group four (n = 5) were injected with venom pre-incubated with *T. alternifolia* methanol extract of two different concentrations. The fifth group (n = 5) of mice was injected with plant extract alone of higher concentration. After 3 h incubation, mice were anaesthetized and blood was drawn by retro-orbital method. Blood was allowed to clot and 1:25 diluted serum was assayed for LDH enzyme activity.

Statistical analysis

Statistical analysis performed using Graph Pad Prism 6. The significance between the groups was calculated by using student unpaired t-test. 'a' represents p <0.05, 'b' represents p <0.01, 'c' represents p <0.001 and 'd' represents p <0.0001. Effective dose (ED_{50}) was the dosage of extract required to produce 50% inhibition was calculated by using regression analysis.

Results

Minimum coagulant dose (MCD) is defined as a dose which induces clotting of human plasma within 60 seconds. *E. carinatus* venom at 1 µg concentration induced clotting of plasma within 1min. *E. carinatus* venom-induced procoagulant effect was evident by sudden coagulation of plasma upon addition of $CaCl_2$. *E. carinatus* venom-induced plasma clot within 14.66 s (VCT) at a concentration of 1 µg of venom (Fig. 1). Normal clotting time (NCT) of human citrated plasma incubated with phosphate buffer saline was 248.33 s. Dose-dependent increase in clotting time was observed when MCD of venom was incubated with various concentration of *T. alternifolia* methanol extract. Venom to extract concentration of 1:60 (w/w) delayed the clotting time up to 100.66 s.

At venom concentration of 5 µg, A α and B β band of fibrinogen was hydrolysed. At venom: extract (w/w) concentration 1:40 completely neutralized the fibrinogen degradation. At low concentration of plant extract the preferential neutralization of Bβ chain degradation over A α band of the fibrinogen was observed (1:15, 1:20, 1:25, 1:30 and 1:35) (Fig. 2).

Haemolytic activity induced by the venom was analyzed by *in vitro* HRBC stabilization method (direct haemolytic activity). The venom of 100 µg/mL was pre-incubated with 100-400 µg/mL of *T. alternifolia* methanol extract. Results were expressed as percentage inhibition of haemolysis, percentage haemolysis by venom was considered as 100%. Dose-dependent decrease in the percentage of haemolysis was observed as the concentration of plant extract was increased. The effective dose (ED_{50})value of *T. alternifolia* for *E. carinatus* venom was 259.86 µg/mL (Fig. 3).

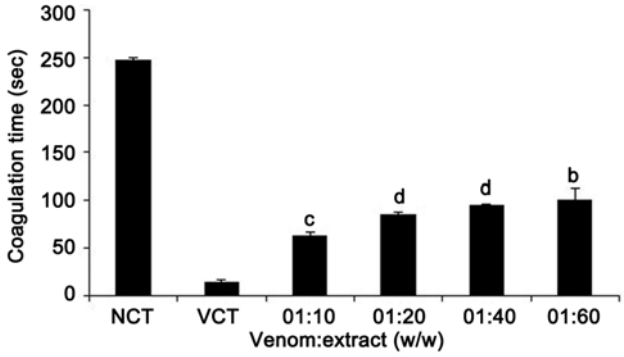

Fig. 1 — Neutralization of *E. carinatus* venom induced procoagulant activity by *T. alternifolia* methanol extract. Values represents mean ± SE of three independent experiments where 'b' representsP < 0.01, 'c' represents P <0.001 and'd' represents P <0.0001. NCT-Normal clotting time, VCT-Venom clotting time

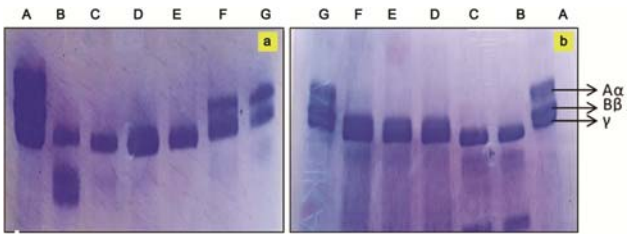

Fig. 2 — Neutralization of *E. carinatus* venom induced fibrinogenolytic activity by *T. alternifolia* methanol extract .a: Lane A- fibrinogen control; Lane B and C- fibrinogen+5µg of *N. naja* venom (F+V); Lane D, E, F and G-F+V incubated with plant extract of different concentration (venom to extract (w/w), 1:5, 1:10, 1:15 and 1:20) b: Lane A-fibrinogen control; Lane B and C-fibrinogen+5µg of *N. naja* venom (F+V); Lane D, E, F and G-F+V incubated with plant extract of different concentration (venom to extract (w/w), 1:25, 1:30, 1:35 and 1:40

Indirect haemolytic assay for PLA_2 activity was measured on agarose-erythrocyte-egg yolk gel plate. Minimum haemolytic halo (MHH) dose for *E. carinatus* venom was 3 µg. About 3 µg *E. carinatus* venom produced 10±2 mm of haemolytic halo which was considered as 1U. Venom-induced haemolytic activity was completely neutralized at 1:15 venom to extract (w/w) concentration (Plate 1).

The lower dose (20 µg) and higher dose (200 µg) of *T. alternifolia* were analysed for toxicity using chick embryo model and was found to be non-toxic with normal growth of the chick embryo were observed. In the mice model, no abnormal behaviour, toxicity and/or pharmacological symptoms were observed throughout the study with a dose of 2000 mg/kg of *T. alternifolia* methanol extract. All mice were alive and normal weight was gained throughout 14 days.

LD_{50} of venom was determined in 6 days old chick embryo having vascularized yolk sac and primitive embryonic heart. LD_{50} value of *E. carinatus* venom was 4.8 µg/egg. ED_{50} of *T. alternifolia* in chick embryo model was 0.98 mg/egg for 2 LD_{50} of the *E. carinatus* venom (Table 1).

LD_{50} value for *E. carinatus* venom was found to be 0.425 mg/kg (12.75 µg/30g) of body weight of mice. Inhibition studies were performed using two different methods; venom was pre-incubated with active plant extracts at two different concentrations and independently injected. The survival time of 3.06 h was observed when a challenging dose of venom was injected. Increase in survival time and the number of survived mice for two different ways of inhibition studies was observed (Table 2).

Saline and plant extracts served as control and were no haemorrhagic corona produced on chick embryo model. Venom at 2 µg concentration produced

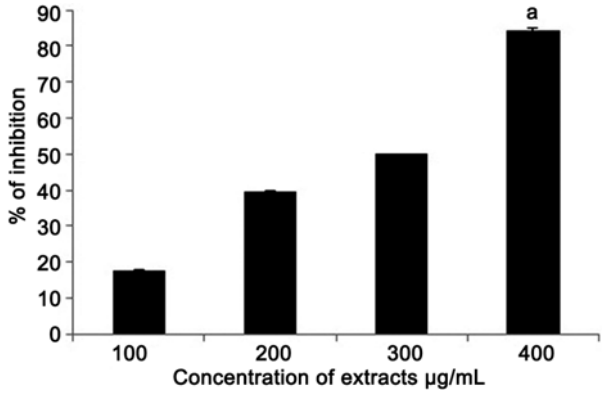

Fig. 3 — *In vitro* HRBC stabilization properties of *T. alternifolia* methanol extract against *E. carinatus* venom induced haemolysis. Values represent % inhibition of haemolysis by *T. alternifolia* methanol extract where 'a' represents $P < 0.05$

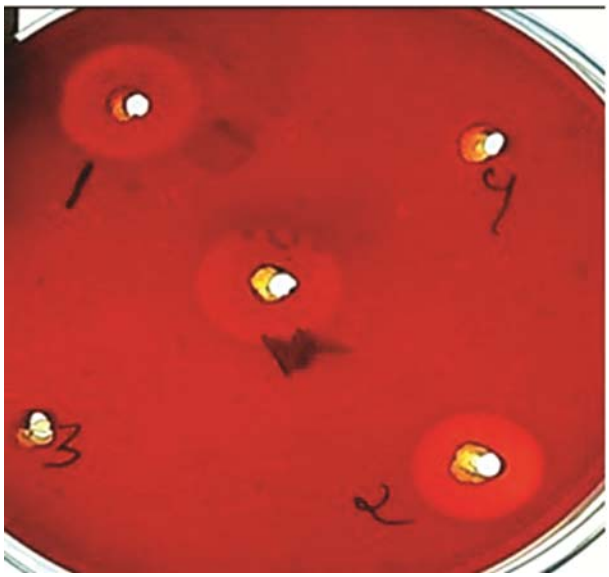

Plate 1 — Neutralization of indirect haemolytic activity induced by *E. carinatus*venom by *T. alternifolia* methanol extract
A and B: (v)-1MHH venom, (1), (2), (3) and (4)-1:5, 1:10, 1:15 and 1:20 venom: extract (w/w) respectively

Table 1 — Neutralization of challenging dose of *E. Carinatus* venom (2 LD_{50}) by *T. Alternifolia* methanol extract in chick embryo model

Group	Venom: extract ratio (w/w)	No of embryo dead	% of survival
I	1:35	5/6	16.6
II	1:40	5/6	16.6
III	1:80	4/6	33.3
IV	1:120	2/6	66.6
V	1:140	2/6	66.6
Saline	100 µL	0/6	100
2 LD_{50}	1:0	6/6	0

Table 2 — Neutralization of challenging dose of *E. carinatus* venom (3 LD_{50}) by *T. alternifolia m*ethanol extract

	3LD_{50} of the venom (h)	Saline (h)	Venom:Methanol extract (w/w) 1:10 (h)	Venom:Methanol Extract (w/w)1:20 (h)
T. alternifolia Pre-incubation Method	03:06a	24b	09:05a	<24 (n =3)
T. alternifolia Separate injection method	03:06a	24b	05:22a	08:45a

'a'- represents all mice dead and 'b' represents all survived

haemorrhagic corona of 2 mm, which was regarded as reference haemorrhagic dose (RHD). Various concentrations (venom: extract of 1:5, 1:10, 1:15 and 1:20 (w/w) of *T. alternifolia* extract were incubated at 37°C for 30 min with 1RHD of venom before application to the discs. At 1:20 venom: extract (w/w) concentration haemorrhagic corona induced by venom was completely inhibited (Plate 2).

Minimum haemorrhagic dose (MHD) of *E. carinatus* venom was found to be 10.00 µg determined by intradermal injection of venom to mice. Saline used as negative control and venom was a positive control. Plant extract of *T. alternifolia* even at 200 µg concentration did not produce any haemorrhagic spot on mice and at 1:20 venom to extract concentration (w/w) completely neutralized the haemorrhagic effects on mice model (Plate 3).

E. carinatus venom produced oedema ratio of 154% at 5 µg venom concentration. For the neutralization studies, 5 µg of venom was pre-incubated with plant extract of two different concentrations. Ratio of 1:10 venom: extract (w/w) oedema ratio reduced to 139.2% and 128% in venom: extract ratio of 1:20 (w/w) (Fig. 4).

E. carinatus venom-induced skeletal muscle damage was quantified by measuring the elevated LDH enzyme activities in the serum. Half the LD_{50} value of *E. carinatus* venom was responsible for the elevation of LDH activity to 4520±6.36 U/L against the control value 568.33±7.6 U/L. Enzyme activity is represented by mean±SE (n=5). For the inhibition studies, venom was pre-incubated with *T. alternifolia* methanol extract of two different concentrations 1:10 and 1:20 respectively (venom: extract, w/w). Plant extract was found to be significantly neutralizing the myotoxic effects in the mice model. Plant extract alone has not altered LDH activity and was observed to be the same as the control value (Fig. 5).

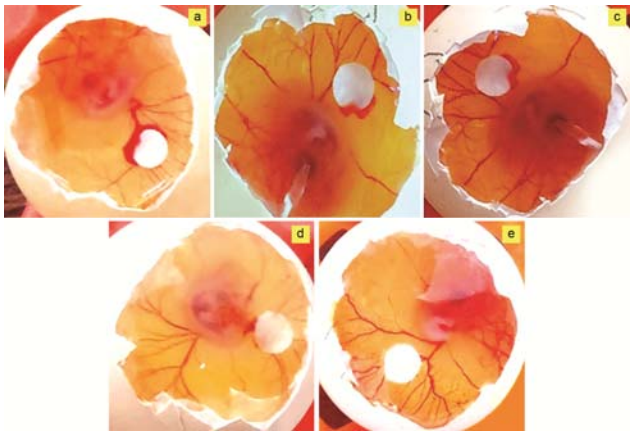

Plate 2 — Neutralization of haemorrhagic activity induced by *E. carinatus*venom by *T. alternifolia* methanol extract on chick embryo model. A- Disc loaded with saline; B- Disc loaded with *T. alternifolia* methanol extract C- 2 µg *E. carinatus* venom; D, E, and F, G- venom pre-incubated with methanol extract of different concentration (venom: extract (w/w) 1:5, 1:10, 1:15 and 1:20)

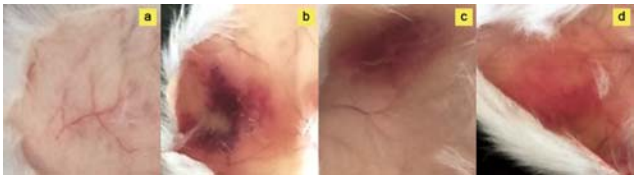

Plate 3 — Neutralization of haemorrhagic activity induced by *E. carinatus*venom by *T. alternifolia* methanol extract on mice model. a- Saline; b- Venom (10 µg); c-Venom + *T. alternifolia* (venom to extract ratio 1:10, w/w); d- Venom + *T. alternifolia* (venom to extract ratio 1:20, w/w)

Discussion

E. carinatus bite is associated with life-threatening local toxicity, haemorrhage and myotoxicity. Studies have revealed that Zn^{2+} metalloprotease, PLA_2s and hyaluronidase induce the progressive local tissue

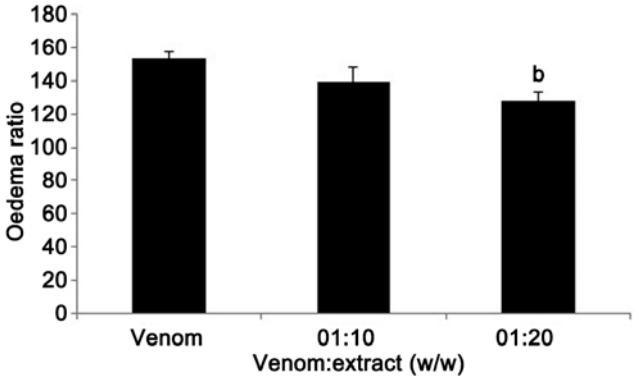

Fig. 4 — Neutralization of oedema induced by *E. carinatus* venom by *T. alternifolia*methanol extract. Where 'b' represents P < 0.01

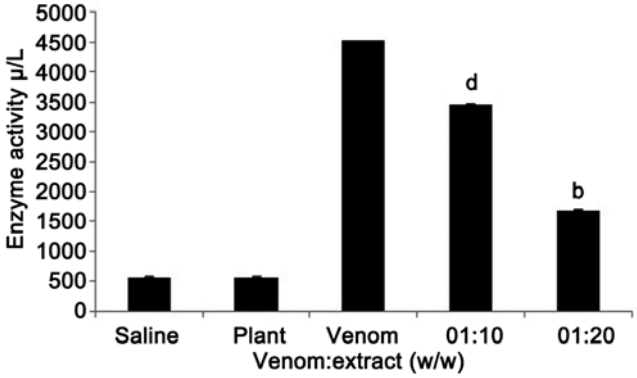

Fig. 5 — Neutralization of myotoxic activity induced by *E. carinatus* venom by *T. alternifolia* methanol extract. Where 'b' representsP<0.01 and'd' represents P<0.0001. LDH activity represented as U/L.

destructions[33,34]. The rapid development of local pathology prevents accessing of anti-serum to the damaged site[4,35].

E. carinatus venom is rich in haemostatically important proteins, which interacts with the proteins involved in the coagulation and fibrinolytic pathway. Venom acute toxin causes life threating coagulation which was the active principle of toxins of viper venom. Venom protease initially acts on the fibrinogen and produce a fibrin clot, which exhibits defibrinating effects (depletion of fibrinogen). Ecarin is a metalloprotease, a very potent prothrombin activator characterized from *E. carinatus* venom[5]. In the present study, it was observed that viper venom was a strong procoagulant as it induced coagulation at 1µg concentration. Inhibition of procoagulant activity induced by the *E. carinatus* was studied up to 1:60 and exhibited moderate inhibition on procoagulant activity induced by the venom. In previous studies, Tamarind seed extract exhibited moderate neutralization on *V. russelii* venom-induced procoagulant activity, whereas haemorrhage effect was completely neutralized by the plant extracts[36].

Fibrinogenolytic activity attributed by the venom is due to either metalloproteinases or serine protease. Majority of snake venom fibrin-(ogen)olytic enzyme characterized was zinc metalloproteinases and belong to the *metazincin* family[37]. *E. carinatus* venom completely degraded Aα and Bβ of the fibrinogen. Complete degradation of Aα and Bβ subunits of fibrinogen will result in the non-availability of fibrinopeptides to form the stable clot. In order to form the stable fibrin mesh, fibrinopeptides Aα and Bβ subunits are essential components[38]. *Vitis vinifera* seed extracts neutralized fibrinogenolytic activity induced by the *E. catrinatus* venom at 1:40 venom to plant extract concentration. Similarly analysed plants were also effective towards the fibrinogenolytic activity.

Phospholipase is most abundant in snake venom, which is present in multiple forms and is responsible for various pharmacological activities. Rapid hydrolysis of erythrocytes was by PLA_2 and haemolysin was reported in viper species[15]. The possible mechanism of inhibition of venom-induced haemolysis may be due to stabilization of the protein by the antidote molecule on the HRBC membrane. In the present study *T. alternifolia,* extract exhibited membrane stabilization of HRBC by interacting with the venom and thereby preventing the haemolysis. Presence of phenolic compound and flavonoids in the plant extracts are the possible inhibitor for HRBC lysis caused by the venom[39]. Indirect haemolytic activity corresponds to PLA_2 activity of venom and which related to the hydrolysis of phospholipid. Inhibitory effects of PLA_2 could be because of direct binding activity or chelation of Ca^{2+} cofactor needed for activities of PLA_2[40]. *Andrographis paniculata* and *Aristolochia indica* were explored for *E. carinatus* venom-induced haemolysis on agarose-erythrocytes gels[14]. Promising inhibition of *E. carinatus* induced haemolysis was obtained at a lower concentration of plant extract.

The main problem associated with the use of rodent for the assay is a large number of animals is required in order to get statistically significant result along with the pain of animal during the experimental period. As a replacement and refinement to rodent experimental models, eggs were used as an alternative set-up[25,29]. In this study, chick embryo was used to study the toxicity of plant, lethal toxicity determination and its neutralization and haemorrhagic studies. Sell's protocol was modified by using 6-day eggs and venom was transferred into eggs, modification of the protocol has made the work less labour intensive. In the case of eggs, extracts were analysed for their efficacy at the various concentration to get effective dose (ED_{50}) for the lethal dose of venom. It was possible to get more information from chick embryo as an alternative model to study and thereby the whole experiment minimized the animal use. Snake venom is well known for its variation in composition, so there is a need to determine LD_{50} values for each batch. Using chick embryo requires less venom to determine LD_{50} values and no ethical approval is required at this stage[26]. This new insensate model has been adopted only in some anti-venom research for lethal toxicity determination and its neutralization studies. *Ophiorrhiza mungos* has been reported for neutralization of *V. russelii* venom-induced lethal toxicity and haemorrhage. Toxicity of the *O. mungos* plant was studied using chick embryo model[15].

Toxic effects attributed to snake bite is a combinatorial effect of toxins and enzymes of the respective venom. Plant extracts are a congregation of pharmacologically important novel molecules, which bind to the toxin/enzyme from the venom and neutralize its effects. Inhibition of the toxicity due to direct binding of the enzyme or chelation of metal ions which are the essential component of enzymatic activity[9,41]. Inhibitory

potential of *T. catharinensis* root extract was reported for *Crotalus durissus terrificus* venom-induced lethal toxicity by different protocols of antagonism mechanisms[17]. Similarly, *T. alternifolia* from the same genus was found to be effective against the Indian *E. carinatus* venom-induced lethal toxicity in chick embryo (*in vitro*) and mice (*in vivo*) models.

Haemorrhage is a common phenomenon in viper and crotoloid bite, where main factor responsible are haemorrhagic metalloprotease and some other venom components. A zinc-dependent metalloprotease is responsible for degradation of extracellular matrix, connective tissue and surrounding blood vessels leading to spontaneous bleeding[42]. Haemorrhagic metalloproteinases disrupt the basement membrane and cause endothelial damages and thereby allowing the blood components to escape from the capillaries. The haemorrhagic toxin acts on local site and also exhibits systemic effects on multiple organs leading to complications like cardiovascular shock, pulmonary bleeding and multiple organ failure[43]. Inhibition of haemorrhagic activity indicates the interaction of plant extracts with metalloprotease. Plant extracts may act as a metal ion chelator in which catalytic site of these enzymes requires metal ions thereby it inhibits zinc-dependent metalloprotease[36]. Plant extract exhibited potent inhibition in both *in vivo* and *in vitro* model that confer that these plants possess potent active constituents, which inhibit haemorrhagic activity.

The local oedema is a common occurrence in snake bite, combined action of metalloprotease and PLA_2 is responsible for the release of endogenous inflammatory mediators. The myotoxic phospholipase acts on skeletal muscles and severely damages the muscles resulting in the elevation of CK and LDH enzyme activities. Various previous studies reported effective neutralization of oedema and myotoxic effects induced by the venom. *Vitis vinifera* seed extract neutralized oedema induced by the *E. carinatus* venom was reported in the previous studies[16]. *E. carinatus* venom mediated elevation of serum enzymes like LDH, glutamic pyruvic transaminase and creatinine kinase was significantly neutralized by aqueous seed extract of *Mucuna pruriens*[44]. *T. catharinensis* aqueous extract represents potent myotoxin inhibitor of *Bothropsjararacussu* venom and isolated myotoxin by *in vitro* and *in vivo* methods[45].

Conclusion

T. alternifolia methanol root extract effectively neutralized local and systemic effects induced by the *E. carinatus* venom (Indian). It can be concluded that the significant neutralization of pharmacological and toxicological properties by *T. alternifolia* root extract appears to be highly promising for further studies like purification and characterization in order to obtain better neutralization and new chemical antidote for *E. carinatus* envenomation. An alternative model curtails the excessive suffering of conventionally used mammalian experimental models. Thus, this study substantiates the use of chick embryo model in venom research. Therefore, *T. Alternifolia* can be used for snake bite treatment in a rural area where anti-venom treatments are limited

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