



Bungarus caeruleus venom neutralization activity of Azima tetraantha Lam. Extract

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ABSTRACT

Azima tetraantha Lam. is native to Africa and India. The plant and its parts are used for treating various ailments including snake bites. The different concentrations of ethyl acetate leaf extract of *A. tetraantha* were used to neutralize the toxic effect of venom through dose dependent enzyme studies and *in vivo* studies. The extract was able to neutralize the 5' nucleotidase, phospholipase A₂, Phosphodiesterase, phosphomonoesterase, acetylcholinesterase and hyaluronidase in a dose dependent manner with concentrations ranging from 43.98–340.1 µg/mL of extract. The extract could retain the lysis of fibrinogen at the concentration of 1:10 (venom: extract, w/w) and also the lysis of lecithin was reduced at a concentration of 1:25 (venom: extract, w/w). The extract was able to neutralize the LD₅₀ of venom in both mice and embryo and also reduce the myotoxic and edema properties of the venom in mice models. The venom did not show any procoagulant and hemorrhagic effect. The leaf extract possess adequate compounds/phytochemicals that could neutralize the toxic properties/activity of the *B. caeruleus* venom.

1. Introduction

Snakebite envenoming is a global public health problem that deserves far more attention from national and regional health authorities than it has been given up until now. The agricultural workers and their family are the most affected by this environmental and occupational disease in some of the rural areas of developing countries in Africa, Asia, Latin America and Oceania [1]. It affects the poor and low-income population of the tropical countries [2]. Antiserum, the only effective therapeutic treatment available and is derived from animal immunoglobulins. The production of antivenom is time consuming, expensive and often is associated with side effects [3]. Numerous plant species are used as folk medicine in many parts of the world where venomous snakes occur. Plant species or their extracts are either used as decoctions or applied directly to the bite area.

Many studies have been carried out to prove the effectiveness of plants and their plant constituents on snake venom neutralization. Aristolochic acid from *Aristolochia radix* was reported to inhibit the enzymatic and pharmacological activities of PLA₂ induced by *Vipera russelli*

venom [4, 5]. Antihyaluronidase activity was reported with *Mimosa pudica* against *Naja naja*, *Vipera russelli* and *Echis carinatus* venoms [6]. Lupeol acetate from roots of *Hemidesmus indicus* significantly neutralized PLA₂ activity induced by Russell's viper [7]. Inhibition of enzymatic activity has been reported with extracts of *Casearia sylvestris* in experimental animals, injected with lethal doses of Bothropic venoms [8]. The *Rauvolfia serpentina* aqueous plant extract neutralized the *in vitro* activities namely procoagulant, direct and indirect haemolytic activities of *Daboia russelli* venom [9]. The *Ophiorrhiza mungos* aqueous root extract neutralized the *Vipera russelli* venom induced lethality and hemorrhage in fertile Chick Embryos [10].

Azima tetraantha Lam. (Family: Salvadoraceae) locally known as "Mulsangu", is a spinous shrub flowering throughout the year found in Peninsular India, West Bengal, Orissa, African Countries and extends through Arabia to tropical Asia. The common names of the plant are Uppimullu, Mulchangan, Needle bush, Yasanku and Kundali in Ayurvedic medicine. In East Africa the powdered roots of *Azima tetraantha* Lam. are applied directly to snake bites and an infusion is taken orally as a treatment. In India and Sri Lanka the root, root bark and leaves are added

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to food as a remedy for rheumatism [11]. The aim of the study was to evaluate the ability of the ethyl acetate extract of *Azima tetraacantha* Lam. to inhibit the enzymatic and pharmacological activities of the *Bungarus caeruleus* venom.

2. Materials and methods

2.1. Venom

The lyophilized venom of *Bungarus caeruleus* was procured from Irula Snake Catcher's Co-operative Society, Kancheepuram, Chennai. The venom was suspended in physiological saline and centrifuged at 2000g for 10 min. The supernatant was used for further analysis and they are stored at 4 °C. The protein concentration was estimated by Lowry's method [12].

2.2. Animals

Swiss Albino Mice (male) with average weight of 25–30g were used as test animals. The animals were obtained from Sri Raghavendra enterprises, were housed in cages at room temperature of 28–32 °C and under a light period of 16–18 h daily. They were fed on standard commercial feed. The experiments were performed with ethical committee clearance (IAEC/NCP/91/2015). The 6th day hatching eggs were purchased from Lakshmi hatcheries, Bangalore.

2.3. Chemicals

5' Adenosine mono phosphate (5' AMP), disodium-p-nitrophenol phosphate, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide, cetyltrimethylammonium bromide, lecithin were purchased from Himedia Laboratories, hyaluronic acid from Rolex laboratories and casein from Sigma Aldrich laboratories, USA. All the other reagents are analytical grade. The blood sample used for experiments was collected from healthy volunteers with their consent.

2.4. Plant collection and extraction

The fresh leaves of *Azima tetraacantha* Lam were obtained in the month of October from Chitradurga district, Karnataka. The plant material has been identified and authenticated from National dietetics and Ayurveda research centre, Bangalore, Karnataka, where a voucher specimen is kept (RRCBI-0736). The plant material was extracted using different solvents and enzyme inhibition studies showed that ethyl acetate extract was more active compared to others [13].

2.5. Inhibition of enzyme studies (dose dependent studies)

Acetylcholinesterase was assayed using acetylthiocholine iodide to the method of Ellman et al. [14], Phosphomonoesterase was assayed by the method of Bessey et al., [15]. Phosphodiesterase was determined according to the method of Lo et al., [16]. Phospholipase A₂ assay was determined according to the acidimetric method of Tan and Tan [17], with little modification. Hyaluronidase activity was assayed turbidometrically by the method of Pukrittayakamee et al., [18]. Protease activity was assayed by the method of Greenberg using casein as substrate [19]. 5' nucleotidase was assayed by the method of Rowe et al., [20]. Inhibition studies were carried out by pre-incubating the venom with different concentrations of extract. The inhibition was expressed in percentage taking activity with venom alone as 100%.

2.6. Procoagulant activity

Recalcification time was determined according to the method of Condrea et al., [21]. 300 µL of fresh human citrated plasma along with 30 µL Tris HCL buffer pH 7.6 was incubated at 37 °C for 1 min. To this

venom (1 µg) was added and clot formation was initiated by adding 30 µL 0.25 M CaCl₂ and time recorded. For inhibition studies the venom was pre incubated with different concentrations (1:10–1:50, venom: extract, w/w) of leaf extract for 30 min.

2.7. Fibrinogenolytic activity

Fibrinogenolytic activity was determined by method of Ouyang and Teng [22]. The reaction mixture containing venom (10 µg) and Bovine fibrinogen (50 µg) was incubated for 1h at 37 °C. 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 PAGE and the protein pattern was visualized by staining with Coomassie brilliant blue.

2.8. Direct hemolysis assay

The activity was assayed through inhibition of *in-vitro* Human Red Blood Corpuscles (HRBC) lysis described previously [10]. The inhibition was carried out by preincubating the venom with varying concentrations of ethyl acetate extract of *A. tetraacantha* (20–100 µg/mL).

2.9. Indirect hemolysis assay (pla₂ activity)

Phospholipase A₂ activity measured indirectly by haemolytic assay on agarose -erythrocyte -egg yolk gel plates by the methods Gutierrez et al., [23]. The venom (2 µg) was loaded to 1.5 mm wells in agarose (0.8% agarose in Phosphate buffer saline) plate containing 1.2% of human erythrocyte and 1.2% egg yolk as a source of lecithin. For inhibition studies venom was pre-incubated with different concentrations of extract of *A. tetraacantha*. The plates were incubated overnight at 37 °C and hemolytic halos were measured.

2.10. Lethal toxicity

The lethal potency in terms of survival times of groups of mice ($n = 5$) injected with venom in the presence and absence of the extract was determined according to the method of Meier and Theakston [24]. Different doses of venom were injected at a constant volume of 200 µL by intraperitoneal route into the mice for the determination of the median lethal dose (LD₅₀). Group I received 3 LD₅₀ doses of venom alone. Group II and III received venom which was pre-incubated with plant extract at different extracts for 30 min at 37 °C. Group IV received extract and venom without incubation. Control mice were injected with saline alone. The survival time was then monitored. The results are expressed as the mean survival time ±SEM.

2.11. Edema inducing activity

The method of Vishwanath et al. [5], was followed. Groups ($n = 5$) of mice were injected in the right foot pads with the venom sample (5 µg) in 20 µL saline and left foot pad with saline. For inhibition studies the venom was pre-incubated with different concentrations of extracts (1:10 and 1:20, Venom: extract, w/w) for 30 min at 37 °C.

2.12. Hemorrhagic activity

The hemorrhagic activity was assayed on mice ($n = 5$) as described by Kondo et al., [25]. For inhibition studies the venom was pre-incubated with different concentrations of extracts for 30 min at 37 °C.

2.13. Myotoxic activity

Myotoxicity was determined on mice ($n = 5$) according to the method of Gutierrez et al., [26] by determining the cytoplasmic marker enzyme lactate dehydrogenase (LDH) in serum samples. For inhibition studies

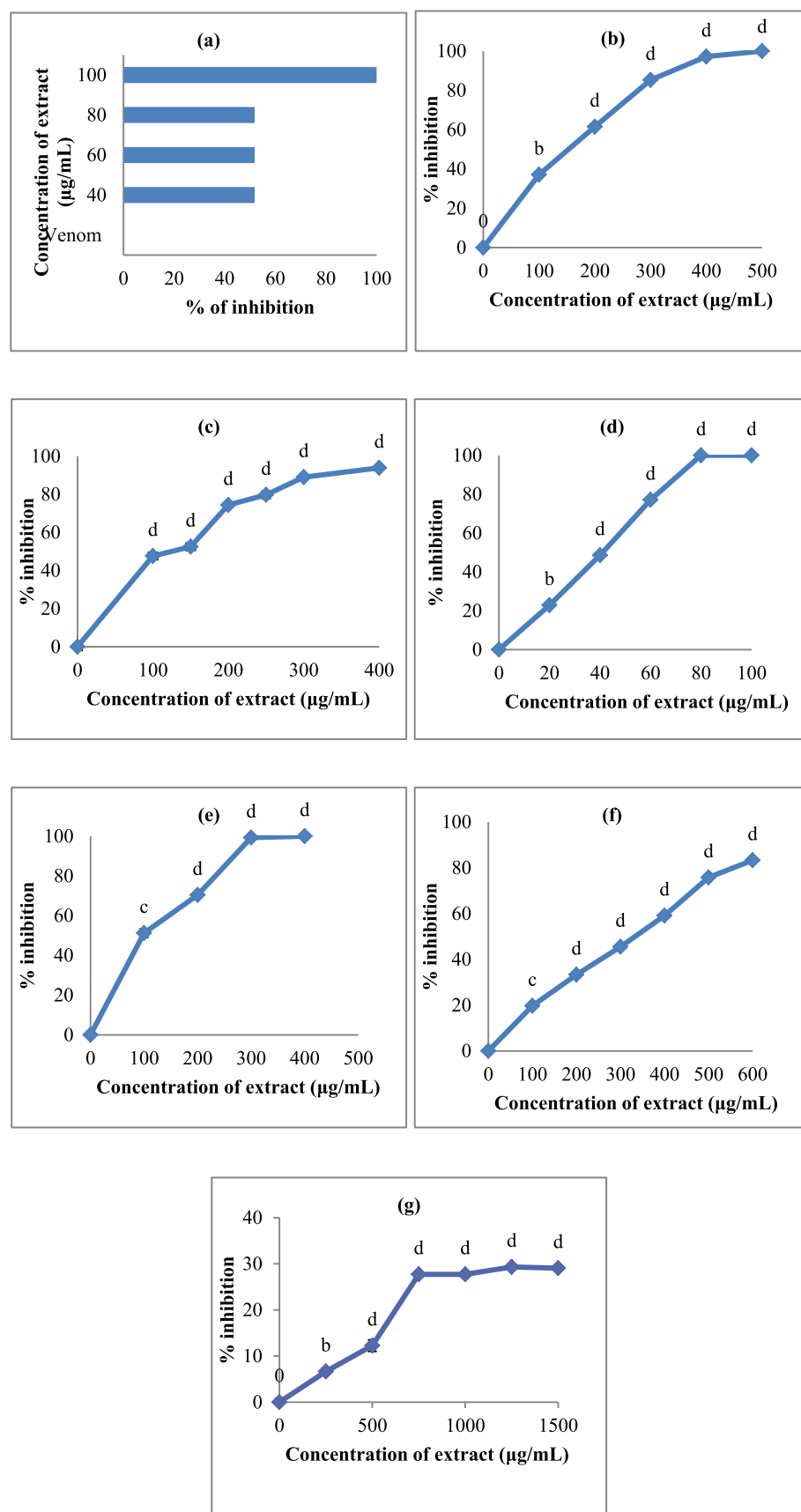


Fig. 1. Inhibition of enzyme activity of *B. caeruleus* venom by *A. tetraantha* leaf extract. (a). 5'-nucleotidase, (b). Hyaluronidase, (c). Acetylcholinesterase, (d). Phosphodiesterase, (e). Phospholipase A₂, (f) Phosphomonoesterase and (g) Protease. Venom was pre-incubated separately with various amounts of extract for 30 min at 37 °C and then the respective assay was performed. Values are mean $\pm$ SEM. ^a P < 0.05 compared to respective control, ^b P < 0.01 compared to respective control, ^c P < 0.001 compared to respective control, ^d P < 0.0001 compared to respective control.

venom was pre-incubated with the extract for 30 min at 37 °C in the ratio of 1:10 (w/w), 1:20 (w/w).

2.14. Acute oral toxicity

Acute oral toxicity of the plant extract was performed according to OECD guidelines 423. Briefly, two thousand milligrams of the test substance per kilogram of body weight was administered to 5 healthy mice by oral gavages. The animals were observed for mortality, signs gross toxicity and behavioural changes at least once daily for 14 days. Body weights was recorded prior to administration and again on Days 7 and 14 (day of termination).

Chick Embryo.

2.15. Chick Embryo preparation

Eggs were prepared with modifications from the standard method [27, 28]. Hatching eggs were slightly cracked to observe the embryo, maintained at 37 °C and relative humidity for the experiment. Discs of 2 mm were cut from Whatman no.1 filter paper using a hand punch. Venom, leaf extract or mixture in a total volume of 2.0 µL was impregnated to each disc and was placed over anterior vitelline vein on the yolk sac membrane. Control tests were carried out using normal saline (0.9%) instead of venom.

2.16. Acute toxicity of leaf extract

Four groups of six embryos each were used per leaf extract dilution (5, 10, 20 and 30 µg/µL) and a total volume of 2 µL was impregnated to each disc with different concentrations were placed over anterior vitelline vein on the yolk sac membrane of the experimental embryos. Control group received 2 µL of saline instead of the extract. The embryos were observed in hourly intervals for 24 h for any lethality. Each test was carried out using triplicate egg preparations.

2.17. Measurement of venom lethality

The venom was tested at different concentrations (0.5–5 µg/µL) for finding the lethal toxicity of viper venom in the 6th day Chick Embryo, using groups of six embryos for each venom dose. A total of 2.0 µL of venom was applied on the disc with different concentrations and was placed on the yolk sac membrane preferably over the anterior vitelline vein. Control groups received normal saline instead of venom. The LD₅₀ was calculated with the confidence limit at 50% probability by the analysis of death occurring within 24h of venom injection. All tests were carried out using triplicate egg preparations.

2.18. Measurement of antivenom activity

Venom sample (3xLD₅₀) was incubated with equal volume of different concentrations (5, 10, 20 and 30 µg/µL) of leaf extract at 37 °C for 30 min before being applied to the yolk sac membrane. Positive control embryo received same amount of venom without leaf extracts. A total of 2.0 µL volumes of each venom and plant extract dilution were expanded to accommodate testing on six eggs. Control tests using 2 µL of saline were also carried out. The embryos were observed at 1, 2, 4 and 6 hourly intervals and the number of survivors at 6h was recorded for the analysis. The death of the embryo was a clear end point with cessation of the heart beat followed by submergence of the yolk sac membrane into the yolk.

2.19. Measurement of hemorrhage

A corona of hemorrhage surrounding discs impregnated with hemorrhagic venoms could be visualized after 2–4 h of incubation at 37 °C. The concentration required to cause a hemorrhagic corona 2mm wide

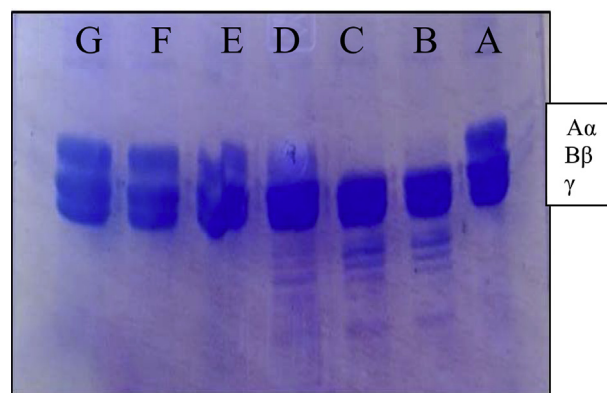


Fig. 2. Inhibition of fibrinogenolysis induced by venom by varying concentrations plant extract. Lane A- Fibrinogen (F) Control; lane B - fibrinogen with 10µg of *Bungarus caeruleus* venom (V), Lane C-G - F + V + A. *tetraclantha* extract (1:2–1:10) venom: extract, w/w).

was accepted as a standard hemorrhagic dose (SHD). Neutralizing or inhibitory activity was determined by incubating one SHD of venom with various concentrations (5, 10 and 20 µg/µL) of the leaf extract at 37 °C for 30 min. The mixture incorporating one SHD, was applied to the disc which was then placed on the membrane as previously described and left for 3 h to form a hemorrhagic corona. The minimum amount of extract required to abolish hemorrhage was recorded as the minimum effective neutralizing dose (MEND). All tests were carried out using triplicate egg preparations.

2.20. Statistical analysis

The results were expressed as average $\pm$ SEM and statistical significance between the groups was determined using student's 't' test. Regression analysis was used to calculate the inhibitory concentration IC₅₀ defined as the dosage of extract necessary to produce 50% inhibition of enzymatic activities. The LD₅₀ dose was also calculated in the same manner for *in vivo* studies.

4. Results

The different extracts of *A. tetraclantha* were evaluated and the ethyl acetate extract was found to be more effective with the initial studies carried out.

The ethyl acetate extract of *A. tetraclantha* inhibited 5' nucleotidase activity of *B. caeruleus* venom completely at 100 µg/mL concentrations (Fig. 1a). The IC₅₀ value of the extract was found to be 55.9 µg/mL. The hyaluronidase enzyme activity of *B. caeruleus* venom was neutralized completely at 500 µg/mL concentrations respectively (Fig. 1b). The IC₅₀ value of the extract was found to be 210.8 µg/mL. More than 80% acetylcholinesterase enzyme activity was inhibited in *B. caeruleus* venom by the crude extract at concentration of 400 µg/mL (Fig. 1c). The IC₅₀ value of the extract was found to be 172.4 µg/mL.

The activity of phosphodiesterase enzyme of venom was completely inhibited at 100 µg/mL concentration of extract (Fig. 1d). The IC₅₀ value of the extract was found to be 43.98 µg/mL.

The phospholipase A₂ activity of *B. caeruleus* was neutralized at 400 µg/mL concentrations of the extract (Fig. 1e). The IC₅₀ value of the extract was found to be 168.9 µg/mL.

The phosphomonoesterase activity was inhibited upto 80% at 600 µg/mL concentration in *B. caeruleus* venom (Fig. 1f). The IC₅₀ value of the extract was found to be 340.1 µg/mL.

The protease activity of *B. caeruleus* was reduced upto 30% at 1500 µg/mL (Fig. 1g). *B. caeruleus* was found to be anticoagulant in nature, even at higher concentration there was no coagulation observed.

The fibrinogen breakdown is mainly because of Zn²⁺

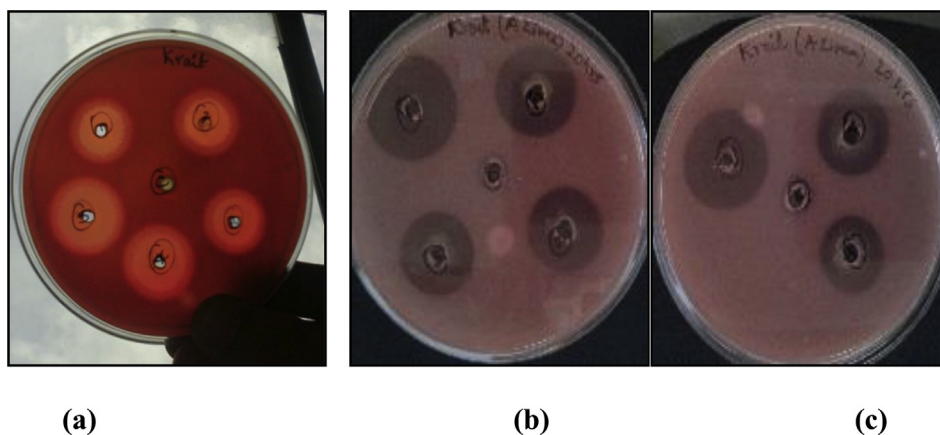


Fig. 3. (a) Hemolytic halos produced by the *B. caeruleus* (1–5 μ g) venoms at different concentrations. (b) & (c) Inhibition of the hemolytic halos produced by the venom by the pre-incubated with different concentrations of active extract (1:5–1:25).

metalloproteases that cleave α and β chain of fibrinogen. With 10 μ g of *B. caeruleus* venom, α chain was cleaved and had no effect on β and γ chain of the fibrinogen. The active extract of the plant inhibited the degradation of the fibrinogen when the venom was pre-incubated with the extract at a concentration of 1:10 (w/w, venom: extract) (Fig. 2). The inhibitory effect on the enzyme might be due to cofactor cleavage.

Different concentrations of *B. caeruleus* (1–5 μ g) venom was incubated in the agarose-erythrocyte-egg yolk gel to determine the amount of venom that produces 11mm diameter halo. It was found that *B. caeruleus* produced 11mm diameter halo with 1 μ g of venom in agarose plate (Fig. 3a).

The LD₅₀ for the *B. caeruleus* was determined by injecting intraperitoneally 2–10 μ g amount of venom into 25–30g of mice in groups and was found to be 200 μ g/kg of mice.

The 3 x LD₅₀ *B. caeruleus* was injected and the survival time was recorded; all mice survived for 24 h when saline alone was injected. In case of pre incubation injections, mice survived for 3 h 28 min and 3 h 50 min at 1:10 and 1:20 (w/w) concentration of *A. tetraclantha* extract. With independent injections, mice survived for 3 h 42 min and 4 h 34 min at 1:10 and 1:20 (w/w) concentration of *A. tetraclantha* extract compared to the 2 h 06 min survival of mice injected with 3 x LD₅₀ venom.

The *B. caeruleus* venom induced a haemolytic halo of 11 mm at a concentration of 1 μ g in agarose-erythrocyte-egg yolk gel. The halo was

reduced to 7mm approximately at a concentration of 1:25 concentration (w/w; venom:extract) with *A. tetraclantha* extract (Fig. 3b & c).

The extract from *A. tetraclantha* reduced the edema caused by *B. caeruleus*. The edema ratio of *B. caeruleus* was reduced from 155% to 136% and 130% with *A. tetraclantha* extract at 1:10 and 1:20 concentration respectively.

The mice injected with *B. caeruleus* venom did not produce the hemorrhagic spot at a higher concentration of 25 μ g. Similar results were observed with other studies as well [29, 30].

With *B. caeruleus* venom, the lactate dehydrogenase enzyme level was elevated to 8827 U/L compared to control. The plant extract did not have any effect on myotoxicity. The levels were reduced to 8230 U/L and 6311 U/L in 1:10 and 1:20 (w/w) concentration of *A. tetraclantha* extract (Fig. 4).

In Chick Embryos, the LD₅₀ was determined by using different amounts of venom (1–5 μ g/ μ L). The LD₅₀ of the *B. caeruleus* was found to be 2.6 μ g/ μ L. With *B. caeruleus* venom (3 x LD₅₀), the first embryo died within 5 h of the experiment and all died by the end of 7th h. The extracts showed neutralizing potential, the embryos survived throughout the experiment at the concentration 20 μ g/ μ L, whereas the few embryos died at 5 μ g/ μ L concentration.

The embryos survived for a period of 24 h in all the concentrations (5, 10, 20 μ g/ μ L) of the leaf extract. They did not induce any toxicity to the embryos throughout the study period.

The hemorrhagic activity of *B. caeruleus* was determined using concentrations of 2–5 μ g/ μ L that produced hemorrhagic corona around the disc placed. No hemorrhagic corona was produced even at higher concentrations with *B. caeruleus* venom.

5. Discussion

Plant extracts are been traditionally used by the folklore to treat various ailments and snake envenomation is one among them. The lack of availability and high cost of antiserum has led the scientific community to look for an alternative.

Animal toxins are complex mixtures of different classes of proteins and peptides such as phospholipases, proteases, esterases and play an important role in envenomation using different mechanisms [31]. 5' nucleotidase is a ubiquitous enzyme, the primary function is to liberate adenosine and other purines which causes platelet aggregation.

Venom hyaluronidase is non-toxic, but it causes tissue destruction locally and results in permanent disability of the victims [32]. The enzyme may lead to circulation of other toxins resulting in systemic effects. The cleavage of hyaluronan may also result in hemostatic disturbances.

Acetylcholinesterase is highly active in elapidae, and one of the key

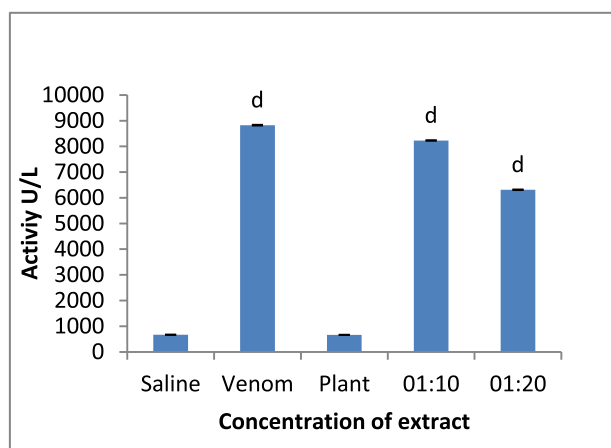


Fig. 4. Neutralization of Lactate dehydrogenase enzyme induced by *B. caeruleus* in mice by the leaf extract of *A. tetraclantha*. Values represent mean $\pm$ SEM. ^a $P < 0.05$ compared to respective control, ^b $P < 0.01$ compared to respective control, ^c $P < 0.001$ compared to respective control, ^d $P < 0.0001$ compared to respective control.

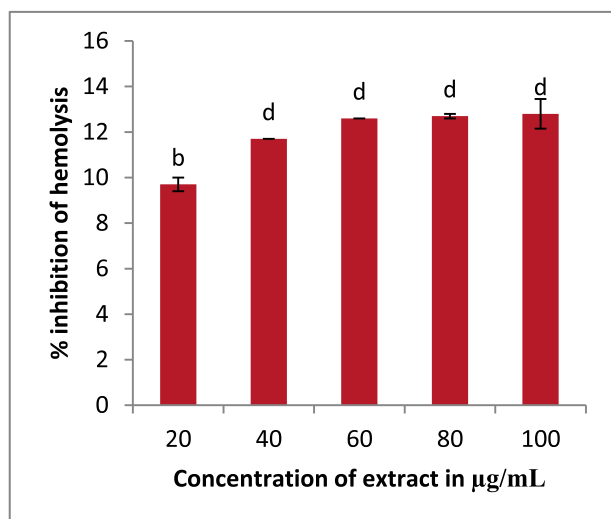


Fig. 5. Inhibition of *B. caeruleus* induced hemolysis of HRBC by *A. tetraecantha* and Values represent mean $\pm$ SEM. ^a $P < 0.05$ compared to respective control, ^b $P < 0.01$ compared to respective control, ^c $P < 0.001$ compared to respective control, ^d $P < 0.0001$ compared to respective control.

proteins involved in nerve impulse transmission. Phosphodiesterases liberates 5' nucleotides from the 3' end of polynucleotides and provide study supply for the 5' nucleotidase of the venom [33]. Phospholipase A₂, one of the key enzymes in prey immobilization and death. They are associated with numerous proteins that bring about the toxicity [34]. The inhibitory effect may be due to the binding or chelating effect of on Ca²⁺ the cofactor for phospholipase A₂.

Phosphomonoesterases are known to non-specifically hydrolyze ribo- and deoxy ribonucleotides such as 5'AMP, 5'-dAMP, 3'AMP, ribose 3-phosphate, ATP. This enzyme along with other enzymes produces purines especially adenosine that causes snake envenomation related effects Dhananjaya and D'Souza [35].

Hemolysis is one of the important lethal effects of the venom due to the presence of phospholipase and hemolysins. With *B. caeruleus* venom, the *A. tetraecantha* extract caused the maximum inhibition of HRBC hemolysis upto 12.6% with 60 µg/mL (Fig. 5). The increase in concentration of the extract did not show any increase in the hemolysis inhibition. The inhibition observed might be due to the stabilization of the membrane proteins in HRBC [10]. Hemolysins and phospholipase A₂ causes hemolysis. The inhibitory effect observed might be due to the chelation of Ca²⁺ cofactor of phospholipase A₂.

Lethality is due to the multifactorial action of the venom and it is dependent on the combined action and the concentration of toxic and non-toxic proteins and peptides. The lethal effect caused by snake venoms is due to the systemic action of neurotoxic, myotoxic, hemorrhage and coagulant compounds [36].

Edema inducing activity of the venom depends upon the pharmacological effect of several toxins. Previous studies on the inhibition of edema activity using plant extracts or purified toxins have shown the inhibitory effect [31, 37, 38].

Myotoxicity occurs due to the presence of myotoxins and myotoxic PLA₂ in the venom [39]. Myotoxins are small proteins and peptides that cause tissue damage. The myotoxicity of the venom was determined by injecting ½ LD₅₀ of the venom intramuscularly. Previous studies reported the ability of several extracts to inhibit myotoxic activities induced by snake venoms [31, 39].

The neutralizing potential observed in the embryos is due to the inhibitory effect of the phytochemical components present in the isolates. However the experiments carried out were not enough prove the neutralization of neurotoxicity caused by the venom which limits the study.

6. Conclusion

Azima tetraecantha extract has been capable of inhibiting the toxic activities of krait as proved by *in vitro* and *in vivo* studies. The plant extracts have been used by the folklore worldwide from centuries and have been able to inhibit or decrease the effect of venom. The scientific evaluation helps to explore the plant properties and its uses. They could be used as first aid on the victims before reaching medical facility.

Declarations

Author contribution statement

Bhavya Janardhan, Vineetha Shrikanth: Performed the experiments. Veena More: Analyzed and interpreted the data. Govindappa Melappa: Analyzed and interpreted the data; Wrote the paper.

Farhan Zameer: Contributed reagents, materials, analysis tools or data.

Sunil More: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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