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# Anti haemolytic potential of aqueous leaves extraction of *Salvia leucantha* cav., against Indian cobra (*Naja Naja*) venom

**Suresh Pullani[1], R Rajendran[2], A Lakshmi Prahha[1*]**
[1] Department of Botany, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India
[2] Department of Botany, Alagappa University, Karaikudi, Tamil Nadu, India

**Abstract**
Snake venoms are complex mixtures of numerous proteins and peptides and extensive interspecific variation in venom composition. Enzymes existing in snake venom hydrolyze protein which leads to tissue gangrene and blood coagulation. Phosphodiesterase A2 causes hemolysis by lysing cell membrane of red blood cells (RBCs). In the present study examined anti-hemolytic activity of aqueous leaves extract of *Salvia leucantha* against Indian cobra (*Naja naja*) crude venom. The aqueous leaves extract of *S.leucantha* was found to have phenomenal anti-hemolytic potential against the crude venom as probed by *in vitro* assay. A rapid inspection of the figure suggests that 200µg/ml of the extract could inhibited about 49% and 800µg/ml of the extract was inhibited the 87% of hemolytic activity of the cobra crude venom. In indirect haemolysis about 12µg of cobra venom produces 14.02mm of haloes. Various concentrations of plant extract were pre-incubated with venom inoculated on the egg yolk agarose gel plate and notably 200µg/ml of extract produces 7.02mm halos, it was calculated inhibition was 50%. The maximum concentration of plant extract 800µg/ml inhibited the 84.80% of indirect haemolysis (PLA2) on egg yolk agarose gel plate. This was concluded that aqueous leaves extraction of *Salvia leucantha* have the ability to inhibiting direct haemolysis and Phospholipase A2dependant hemolysis.

**Keywords:** snake venom, anti-venom, neglected tropical diseases, envinomation, haemolysis, phospholipase

## Introduction
Snake bite is a common and frequently devastating environmental and occupational disease, especially in rural areas of tropical developing countries and is responsible for tens of thousands of deaths and disabilities every year. The World Health Organization has added snakebite to the list of Neglected Tropical Diseases in 2009. India is estimated to have the highest snakebite mortality in the world. World Health Organization (WHO) estimates place the number of bites to be 83,000 per annum with 11,000 deaths. India therefore contributes to a significant proportion of global snake bite deaths. Snake venoms are the most complex of all natural venoms and poisons. Snake venom neurotoxins block/excite peripheral neuromuscular junctions by acting at various sites and bind to their receptors with high affinity, making reversal of paralysis by anti-venom implausible. Anti-venom, prepared by immunizing horses or sheep with venom from snakes is the only medically accepted remedy for systemic snake envenomation. All Indian anti-venoms are polyvalent, that is, they are effective against all the Big Four common venomous snakes of India. However, the high cost of generating antibodies in horses and side effects, such as serum sickness, are bona fide problems.
Snake venoms are complex mixtures of numerous proteins and peptides and extensive interspecific variation in venom composition poses major challenges for the development of generic (i.e., pancontinental) snakebite treatments (Casewell, N. R. *et al.,* 2014 [2] and Tasoulis, T. & Isbister, G. K.2017) [3]. Current therapies, known as anti-venoms, consist of polyclonal immunoglobulins purified from the plasma/serum of large animals (e.g., equines, ovines) hyper immunized with snake venoms. Because of the specificity of the resulting immunoglobulins towards the toxins present in the venoms used in manufacture, anti-venoms typically have limited efficacy against envenoming by different snake Species (Williams, D. J. *et al* 2011) [4].
*Naja naja* venom is highly lethal in nature containing high amount of systemic toxins. It is a mixture of neurotoxins (pre/post synaptic), cardiotoxins, myotoxins and locally acting enzymes (Girish *et al.,* 2004; Satish *et al.,* 2004) [5, 6]. Venoms contain proteins, lipids, amino polysaccharides, amines, quinines, neurotransmitters and other compounds, and are capable of causing many effects. Elapid venoms have higher concentrations of esterase, such as acetylcholinesterase, that exerts effects on the nervous system (Dey A, De JN 2011) [7]. Proteolytic enzymes like trypsin are account for much of the digestive reactions of snake venoms. Phospholipases A and B degrade lipids to free fatty acids and can cause damage to the cell membrane causing lysis and apoptosis (Chavanayarn C *et al.,* 2012) [8]. PLA2 also displays various pharmacological activities such as neurotoxicity, hemolytic activity, myotoxicity, anticoagulant and antiplatelet activities.
Since ancient times, plants have been used for treatment of various diseases. The traditional systems of medicine, together with folklore systems, continue to serve a large portion of inhabitants, particularly in rural and tribal areas despite the advent of modern medicine (Fabricant DS, Farnsworth NR, 2001) [9]. There are some critical issues with ASV, the production of which started 100 years ago in India. The potency of the presently available ASV is less than what it was prior to 1950's. The main issues with ASV in actual clinical practice are species specificity, difficulty in availability, affordability and ideal storage conditions. One of the principal drawbacks of the immunotherapy is the issue of specificity. There is a huge species variation with

current taxonomy identifying one, four and eight species of Russell's viper, cobras and kraits, respectively. So the variable composition and antigenic reactivity of the venom restricts the use of a particular ASV to a geographical area with relevant specificity. Venom variation, low potency, bites by other species could be responsible for the reported failure of polyvalent ASV in countering the venom effects in India. The use of plant remedies to treat snake bite victims in rural areas and poor communities in developing countries is a common practice (Kuntal, Das 2009) [10]. The natives whose majority are rural farmers come in contact with snakes during their farming engagements. Due to high cost of hospital treatment and unavailability of antivenins, most often, the rural people find it more convenient to consult native doctors who are acclaimed for curing snake bite patients. There are many anecdotal evidences which indicate that plant remedies used by native doctors are effective in curing snake bites, and there appears to be a high rate of survival among snake bite patients in advanced clinical stages of venom toxicity (Hasson, S. S., *et al.*, 2010) [11].

## Materials and Methods

### Collection of Venom

Lyophilized Indian cobra (*Naja naja*) venom was obtained from Irula snake catcher's Industrial co-operative society limited (Chennai, India). The venom was dissolved in 0.9% saline and centrifuged at 5000g for 10 min and the supernatant was used for the study. All the reagents and solvents were of high quality analytical grade and were purchased from Sisco Research Laboratory Pvt Ltd/Hi Media, Mumbai.

### Collection of Plant and Preparation of Sample

Fresh and young leaves of *Salvia leucantha Cav.* was collected from the Kodaikanl, Tamil Nadu, in the month of July, 2019. The leaves of plant material was shade and air dried, then leaf material was coarsely powdered and 80g was used for extraction, extracted by refluxing with aqueous (Sterile distilled water) solution (60–80 °C for 72 h). The collected extraction filtered with Whatmann No.1 filter paper and was concentrated in vaccuo and kept in desiccators at room temperature for further use.

### *In vitro* Haemolytic assays

### Direct Haemolysis Assay

The hyposaline-induced haemolysis was modified in the present study by venom-induced haemolysis. Blood was collected from healthy human volunteers by vein puncture and Ethylene di tetra amine (EDTA) was used as an anticoagulant. The collected blood was washed three times with saline and 1% Human red blood cells (HRBC) was prepared. Lyophilized venom of *Naja naja* was dissolved in physiological saline solution to make a stock solution of 100µg/ml. Then 1 ml of venom (100µg), 1ml of phosphate buffer (pH 7.2) and 1ml of 1% HRBC was taken in various tubes. To these tubes different concentrations of the aqueous extracts of *S. leucantha* leave extract (50, 100, 200, 400, 600 and 800µg/ml) were added. The control samples were mixed with extract free solutions. The mixtures were incubated at 37 °C for 30 mins and centrifuged at 1000 rpm for 3 mins. The absorbance of the supernatant was measured at 540 nm using spectrophotometer (Rajendran K, *et al.*, 2010) [12]. The percent inhibition of haemolysis was calculated according to the equation:

$$Inhibition\ \%of\ Hemolysis = \frac{Control\ OD - Treated\ OD}{Treated\ OD} \text{X100}$$

### Indirect Hemolysis Assay (PLA2 activity)

PLA2 inhibition was evaluated using egg yolk as substrate in 1% agarose plates according to the method described by Gutierrez *et al.*,. (1988) [13]. 50µg of *N. naja* venom was pre-incubated with various concentrations of plant extract for 1 h at 37 ◦C. The pre-incubated samples were then loaded into 3 mm diameter wells of 1% agarose plates containing 0.6% egg yolk and 5 mM $CaCl_2$ followed by overnight incubation at 37 ◦C. The PLA2inhibition was calculated by measuring the zone of clearance in the presence and absence of plant extract. PLA2activity of venom in absence of plant extract served as control.

## Results and Discussion

Cobra venom contain phospholipase enzyme, which acts on membrane associated phospholipids liberated lysolecithin, which act on the membrane of human red blood cells and causes lysis of HRBC. *S.leucantha* aqueous extract acts on phospholipase enzyme, which neutralizes the haemolysis activity. Direct addition of snake venom into Human RBC produced hemolysis result in release of hemoglobin. Various concentrations (50,100,200,400,600 and 800µg/ml of aqueous extract was pre-incubated and used for the experiment. Absorbance of supernatant was measured after centrifugation. Toxic control (venom only) group showed high level of absorbance since it produce maximum hemolysis. Co-administration with Aqueous leaves extract of *S.leucantha* significantly reduced the absorbance which denotes the inhibition of hemolysis. 50µg/ml extract inhibited 6.85% of haemolysis. 200 µg/ml inhibited 49.71% and 800 µg/ml of plant extract showed 87.94% anti haemolytic potentiality against Indian cobra crude venom (Fig: 1).

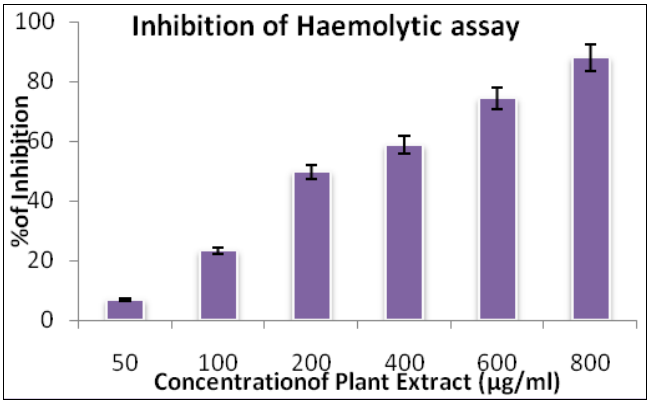


**Fig 1:** Inhibition of Haemolysis

Plant extracts constitute rich sources of novel compounds with a variety of pharmacological activities. The aqueous extract of the plant reduced the mortality and significantly inhibited the onset, and severity of neurotoxic signs induced by the venom. Since the traditional haelers sometimes administrated these agents by making it into a paste and tying them over incisions made at the point of bites, it is possible that the toxic enzymes might be neutralized

through the process. Medicinal plants are an important source of bioactive compounds that assist directly in the handling of ophidian envenomation, or ultimately, as supplements to conventional serum therapy. Thus, plant extracts are a valuable substitute, used either alone or in combination with other agents, when antisera are not available in emergency situations (Pullani. S and Prabha AL, 2020) [14].

In the assessment of Phospholipase A2, *N.naja* venom was able to produce haemolytic haloes in agarose-sheep erythrocytes gels. About 12µg of *N.naja* venom produces 14.02mm diameter haemolytic halo (Table1). This indicates that *N.naja* venom have the enzymes (Phospholipase A2) that has the ability to lyse sheep RBC's. Aqueous extract of *S.leucantha* was capable of inhibiting Phospholipase A2dependant hemolysis of sheep RBC's induced by *N.naja* venom in a dose dependant manner. It was observed that 800µg/ml of plant extract was remarkably inhibited the halos 2.13mm in diameter with inhibition 84.80%. Inhibition of halos and percentages were showed in the Table2 & Fig2. Anti-snake venom properties of *Schizolobium parahyba* (Caesalpinoideae) aqueous leaves extract and reported that the aqueous extract of *Schizolobium parahyba* showed potent anti-snake venom activity (Mendes *et al*. 2008) [15].

Previous *in vitro* studies confirmed that the aqueous leaves extraction of *S.leucantha* remarkably neutralize the Indian cobra venom enzymes such as hyaluronidase, acetylcholinesterase, 5'nucleotidase and Phosphodiesterase (Suresh Pullani, R Rajendran, and A Lakshmi Prabha, 2021) [16].

**Table 1:** Effect of *N.naja* venom induced haemolysis on egg yolk agarose gel plate

| S.No | Concentration of Venom (µg) | Haleos in mm |
|---|---|---|
| 1 | 1 | 2.9 |
| 2 | 2 | 3.15 |
| 3 | 4 | 5.47 |
| 4 | 6 | 8.59 |
| 5 | 8 | 10.09 |
| 6 | 10 | 12.15 |
| 7 | 12 | 14.02 |

Enzyme inhibiting and protein binding properties have been associated with chemically active compounds of flavonoids, polyphenols, terpenoids, xanthene etc. The phytochemicals also inhibit PLA2 activities of viper and cobra venom. Phenolics, especially polyphenols, like some tannins bind proteins, acting upon components of venom directly and disabling them to act on receptors. They could also act by competitive blocking of the receptors (Lans *et al.*, 2001) [17]. Tannic acid has been found to be a potent inhibitor of hyaluronidase. Pentacyclic triterpenes, betulin and betulinic acid extracted from *Betula alba* have demonstrated activity against PLA2 (Soares *et al.*, 2005). Edunol, a pterocarpan isolated from *Harpalyce brasiliana* was found to be anti proteolytic and an inhibitor of PLA2. A triterpenoid saponin from *Gymnema sylvestre*, potassium salt of gymnemic acid has inhibited ATPase induced by *Naja naja* venom (Kini and Gowda, 1982a) [20].

**Table 2:** Effect of Aqueous leaves extraction of *S.leucantha* + venom induced haemolysis on egg yolk agarose gel plate

| S.No | Concentration of Plant Extract (µg/ml) | Haloes (diameter in mm) | % of Inhibition |
|---|---|---|---|
| 1 | 25 | 10.01 | 28.60 |
| 2 | 50 | 9.53 | 32.02 |
| 3 | 100 | 8.75 | 37.58 |
| 4 | 200 | 7.01 | 50 |
| 5 | 400 | 6.52 | 53.49 |
| 6 | 600 | 3.2 | 77.17 |
| 7 | 800 | 2.1 | 84.80 |

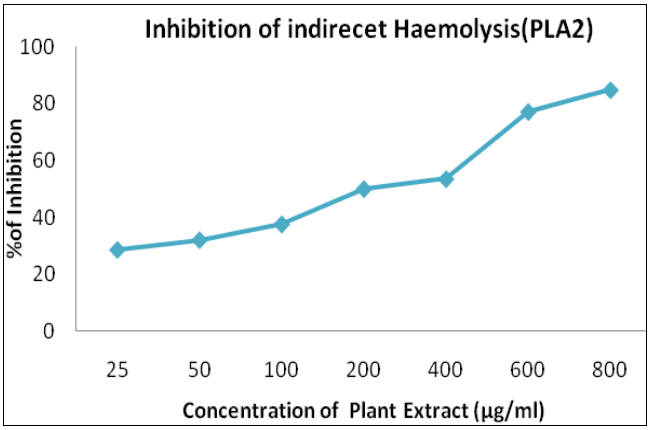


**Fig 2:** Inhibition of Indirect Haemolysis

## Conclusion

The anti-venom property determination reveals that the aqueous leaves extract of *S.leucantha* showed a significant activity against the *N.naja* venom. It was effective in neutralizing the hemolytic and toxic enzymes of venom PLA2 activity. The previous results showed that plant extracts were capable of inhibiting acetylcholinesterase, protease, direct hemolytic, phospholipase, procoagulant activities. Hence, the presence of these multiple bioactive (anti-snake venom) compounds in the extract could have contributed for its efficient anti-venom activity in the present study. Based on the finding of the present study, it is demonstrated that aqueous extracts of *S.leucantha*, possess anti-haemolytic activity against *Naja naja* venom.

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## Conflict of Interest

None

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