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In vitro neutralization of *Naja naja* venom enzymes by using aqueous leaves extraction of *Salvia leucantha* Cav.

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Abstract
Treatment of snakebites envenomings is critically dependent on the availability of effective anti-venoms. Medicinal plants have been extensively studied in order to obtain an alternative treatment. The present *in vitro* neutralization study aims to provide a scientific explanation for the use of aqueous leaves extraction of *S.leucantha* against snakebite activity on toxic venom enzymes like hyaluronidase, acetylcholinesterase, 5'nucleotidase and phosphodiesterase, from Indian cobra *(Naja naja)* venom. Maximum inhibition of Hyaluronidase 72.91%, Acetylcholinesterase 71.76%, 5'nucleotidase 76.48% and Phosphodiesterase 71.16% observed at 100µg/ml concentrations. This observations proved that the aqueous extract has the anti venom efficacy against venom enzymes.

Keywords: Snake venom, enzymes, hyaluronidase, acetylcholinesterase, anti venom, salvia, phosphodiesterase

Introduction

Snake venom is essentially highly modified with saliva. Snake venom is complex mixtures of proteins, peptides and metal ions (Saviola *et al.,* 2015) [1]. The snake venom contains many biological active compounds, which act as several biological resources such as Neurotoxic, Cardiotoxic, Cytotoxic and Haemotoxicity (Koh *et al.,* 2006; Doley and Kini, 2009; Y *et al.,* 2014) [2, 3]. Many multiple roles were involved in snake venom proteins such as killing or immobilizing prey as well as assisting in the digestion process. Approximately 20 different types of toxic enzymes were involved in snake venom (Aird, 2002) [4]. The most of the common enzymes in snake venom were phospholipases A2 (PLA2), serine proteinases, metalloproteinases, acetylcholinesterases (AChEs), l-amino acid oxidases, nucleotidases (5'-nucleotidases, AT Pases, phosphodiesterases and DNases) and hyaluronidases. In most cases, the snake venom contains large amount of abundant source for the enzymes. The Phospholipase A2 (PLA2), Metalloproteinases and Serine proteinases are important proteins in snake venom. The phospholipases A2, snake venom metalloproteinase's and snake venom serine proteinases were being the most abundant and most frequently associated with the clinical symptoms of envenoming.

The anti-venom immunotherapy is the only effective treatment for the snakebite (Gutiérrez, 2015) [5]. Anti-venom first developed by Calmette *et al.,* (1895) [6] aimed to neutralize venom toxins and was experimented against Indian Cobra (*Naja naja*). The snake envenomation is frequently treated with horse or sheep (Hasson *et al.,* 2010) [7]. The anti-venom might be against single venom to produce monovalent anti-venom or against the pooled venoms of several species of snake to produce polyvalent antivenom. The first choice of treatment should be monovalent or polyvalent anti-serum. In India polyvalent antiserum only using for the treatment of snakebite. However, many of the deaths in the world were anti-serum therapy is not accessible (Chippaux, 1998) [8], is not immediately available, limited availability of the antiserum in remote areas and antiserum does not provide protection against neutralizing local effects, such as hemorrhage, edema and myonecrosis (Yingprasertchai *et al.,* 2003; Girish and Kemparaju, 2005; Murari *et al.,* 2005; Oliveira *et al.,* 2005; Otero-Patiño *et al.,* 2012) [9, 33, 10, 11, 12]. Several tribal and other populations traditionally used some specific plants for the treatment of snakebite. Interestingly, these kinds of plants were used for all kind of snakebite with diversed pathogenesis. This observation motivated in to decode the molecular mechanisms involved traditional plant (*Clematis gouriana* Roxb. Ex DC) used for the treatment of snakebite (Mukherjee and Green, 2012) [13].

Polyvalent anti snake venom is the only therapeutic agent available throughout the world. Major drawback of serum therapy is its prohibitive cost and chance that victims may avail untimely medical care because of remote locations when bitten. Generally anti snake venom serum scarce commodity in quality and supply to rural areas because of requirement of ideal storage condition which may not be possible in rural area. Apart from these there is a great chance that it may also cause hypersensitivity. There are various medicinal plants which have been against snake envenomation in folk and traditional medicines. In ancient Indian system of *Ayurveda*, there are many plants recommended in the management of snakebites. Most of these traditional, ethno medicinal herbs are utilized without any scientific validation. Therefore, this type of treatment needs through scientific investigation. These herbs are lacking with proper scientific validation, but may prove as an alternative to the anti snake venom when studied thoroughly. (Sandeep *et al* 2012) [14]

Many medicinal plants were traditionally reported against the treatment of snakebite in worldwide (Molander *et al.,* 2012) [15]. The plants based medicines were affordable for the socioeconomically challenged people in tropical areas. Plants used in traditional medicine systems are a proven source for finding useful compounds against the necrosis acting enzymes. Some examples are the *alkaloid aristolochic acid* from Aristolochia sp. (Aristolochiaceae) (Girish and Kemparaju, 2006) [16], *rosmarinic acid* and *4-nerolidylcatechol hydroxylated phenolic* compounds isolated from Cordia verbenacea (Boraginaceae) and Piper species (Piperaceae), respectively (Carvalho *et al.*, 2013) [17]. The present study discussed about neutralization efficacy of leaf extraction of *S.leucamntha*.

Materials and Methods

Collection of Venom

Snake Venoms The freeze-dried lyophilized venom of Cobra (*Naja naja*) was obtained from Irula snake catchers Industrial cooperative Society Limited Chennai and was stored at 4 °C. The Stock solution was prepared by dissolving 1mg of lyophilized venom in 1ml of sterile saline (1mg/ml).

Chemicals

5′ Adenosine mono phosphate (5′ AMP), disodium-pnitrophenol phosphate, L-leucine, diansidine hydrochloride, horseradish peroxidase, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide, hyaluronic acid, cetyltri methyl ammonium bromide, lecithin were purchased from Himedia Laboratories (India) and casein from Sigma Aldrich Laboratories (USA). All the other reagents were of analytical grade.

Collection of Plant and Preparation of extracts

Fresh and healthy leaf material of *S. leucantha Cav.*, was collected from the Kodaikanl, Tamil Nadu, in the month of July. The leaves of plant material was washed with sterile distilled water and shade dried for 10 days, about 250 g of dried material coarsely powdered leaf material was used for extraction, extracted by refluxing with aqueous (Water) (60–80 °C for 72 h). Then the extracts were concentrated in vaccuo and kept in desiccators at room temperature for further use.

In vitro enzyme assays

Hyaluronidase

Hyaluronidase assay of crude venom was determined turbidometrically by the method of Pukrittayakamee *et al.* (1988) [18]. The assay mixture contained buffer Tris–HCl (pH 8.0), 50 µg of hyaluronic acid (0.5 mg/mL in buffer) and enzymes in a final volume of 1.0 mL. The mixture was incubated for 15 minutes at 37 °C and the reaction was quenched by the addition of 2 mL of 2.5% (w/v) cetyltri methyl ammonium bromide in 2% NaOH (w/v). The absorbance was read at 400 nm (within ten minutes) against a control solution containing 1 mL of the same buffer and 2 mL of 2.5% (w/v) cetyltri methyl ammonium bromide in 2% NaOH (w/v). Turbidity reducing activity was expressed as a percentage of the remaining hyaluronic acid, taking the absorbance of a tube in which no enzyme was added as 100%. One unit was defined as the amount of enzyme that provoked 50% turbidity reduction. Specific activity was defined as turbidity reducing units per milligram of enzyme. For the inhibition studies, venom was preincubated with extracts for 30 minutes at 37 °C.

$$\% \text{ of Inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \text{ X100}$$

Acetyl cholinesterase

Acetyl cholinesterase inhibition assay was carried out according to the modified method of Ellman *et al.* (1961) [19]. 200 µg of venom (1 mg/mL) was pre-incubated for 1 h with different concentrations (5, 10, 25, 50, 75 and 100µg/mL) of plant extract and supernatant was added to the assay mixture which consists of 100 µL of 75 mM acetylcholine idodate in 1 mL of phosphate buffer. The activity was measured by taking the absorbance at 412 nm. Venom without plant extracts was considered as control or 100% activity. Percentage of inhibition was calculated by following formulae

$$\% \text{ of Inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \text{ X100}$$

5′ nucleotidase

5′ Nucleotidase was assayed by the method of Rowe *et al.* (1980). The substrate solution contained 1 mL of Tris–HCl buffer (pH 8.0), 0.1 mL of 0.1 M magnesium chloride and 0.8 mL of 0.15% 5′AMP followed by 0.25 mL of 0.1% crude venom and incubated at 37 °C for 15 minutes. At the end of incubation time, the reaction was quenched by adding TCA and filtered. The filtrate was assayed for inorganic phosphate by the method of Fiske and Subbarow (1925) at 625 nm using potassium dihydrogen phosphate as standard. In this analysis, one unit of enzyme activity was defined as the amount that yielded 0.01 µmole of inorganic phosphate/ minute under the experimental conditions. For the inhibition studies, venom was pre-incubated with different concentrations of the extracts for 30 minutes at 37 °C. Inhibition was calculated as follows

$$\% \text{ of Inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \text{ X100}$$

Phosphodiesterase

Phosphodiesterase activity was determined by a method modified from Lo *et al*. 1966. The assay mixture contained 0.1 mL of venom solution, 0.5 mL of 0.0025 M Na-pnitrophenyl phosphate, 0.3 mL of 0.01 M $MgSO4$ and 0.5 mL of 0.17 M Tris–HCl (pH 8.0). The absorbance was measured at 400 nm. Phosphodiesterase activity was expressed in nanomoles of product released/minute. Molar extinction coefficient at 400 nm was 8100 Cm^{-1} M^{-1} [34]. For the inhibition studies, the venom was preincubated with the extracts for 30 minutes at 37 °C.

$$\% \text{ of Inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \text{ X100}$$

Results and Discussion

Plant extracts are being traditionally used in the treatment of snakebite envenomations, especially in remote areas where hospital facilities are limited (Borges *et al.*, 2001) [23]. Though reports indicate that many of these herbal plant formulations exhibit anti-venom activity (Gomes *et al.*, 2010) [24], the molecular basis of the anti-venom activity of several of these herbal extracts is still elusive. During the last few years there has been an increasing interest in the study of medicinal plants and their traditional use of different parts of India. In the recent years number of reports on the use of plants in traditional healing by either tribal people or indigenous communities of India is increasing (Namsa *et al*., 2009; Upadhyay, Dhaker, Kumar, 2010) [25, 26]. The herbal medicines are mostly administered in the form of juice, decoction, paste or powder, prepared in a crude method from different plant parts such as root, bark, leaves, flowers, fruits, seeds and whole plant (Sarada *et al*., 2008) [27].

Inhibition of Hyaluronidase activity

Hyaluronidase is a spreading factor which facilitates the venom distribution at the site of bite and cause local tissue damage (Girish *et al*., 2004b) [28]. Anti-venom is less effective in preventing local tissue damage (Meenatchisundaram and Michael, 2009) [29]. Different phenolic or flavonoid compounds and alkaloids isolated from various plant sources were found to inhibit snake venom Hyaluronidase and their spreading activity (Kuppusamyand Das, 1991; Pessinietal., 2001; Girish *et al*., 2004a; Girish and Kemparaju, 2005) [30, 31, 32, 33]. Different concentrations of aqueous extract 5, 10,25,50,75 and 100µg/ml was used for the assessed the activity against snake venom. The maximum inhibition was recorded as 72.91% at 100µg/ml concentration and the minimum was recorded at 5µg/ml with 38.22%. Results were showed in the Fig1.

Inhibition of Acetyl cholinesterase activity

The aqueous extract of the plant was taken in different dilutions starting from 5µg to 100 µg with triplicate experiments. Maximum of Acetyl cholinesterase inhibition 71.76% was occurred at 100 µg concentration of venom and aqueous extract of plant respectively. The activity was calculated in terms of percentage of inhibition compared to venom pre-incubated with different amounts of plant extract and venom with substrate. The enzyme reaction was observed for every 10 minutes intervals at 412 nm results were showed in the Fig2.

Inhibition of 5' nucleotidase activity

To assess the *in vitro* inhibition of 5'nucleotidase, the venom degrades the substrat into peptide precipitation could be observed at 625 nm. Maximum inhibition 76.48% was occurred at 100 µg concentrations of venom and aqueous extract of plant respectively. From the results it was observed that increased amount of plant extract could increase the inhibition of 5'nucleotidase of cobra activity results showed in the Fig3.

Inhibition of Phosphodiesterase

Phosphodiesterase inhibition was calibrated by liberation of inorganic phosphate with of positive control of venom (200 µg) and substrate as ATP (10 µm). Different concentrations of venom and substrate were used for this reaction. The same concentration of venom 200 µg with different amounts of active aqueous extract from the plant 5 µg to 100 µg was pre-incubated for the reaction. Maximum inhibition up to 71.16% has been noted at highest amount of plant extract concentration Fig4.

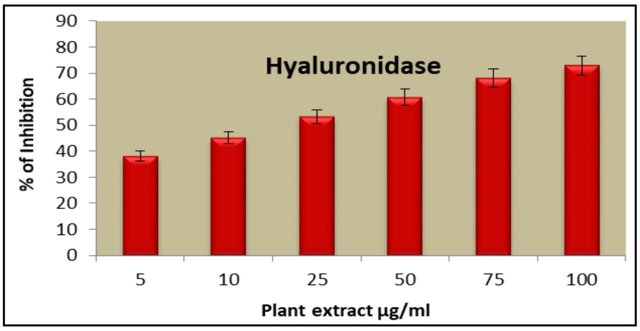

Fig 1: Inhibition of hyaluronidase Activity

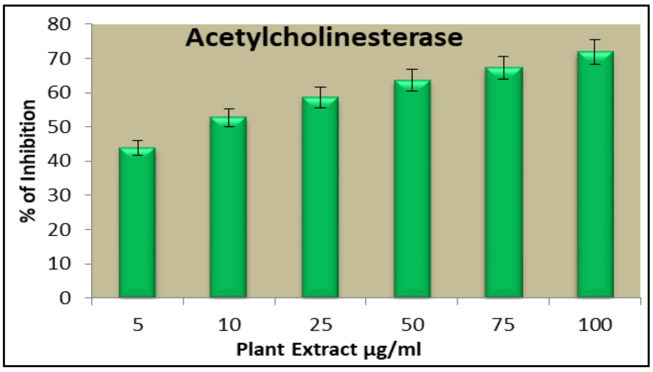

Fig 2: Inhibition of Acetylcholinesterase Activity

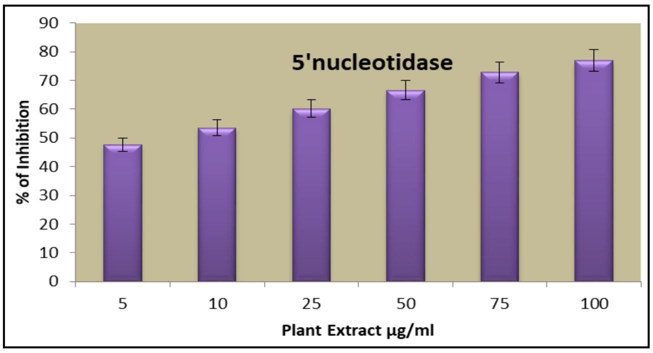

Fig 3: Inhibition of 5'nucleotidase Activity

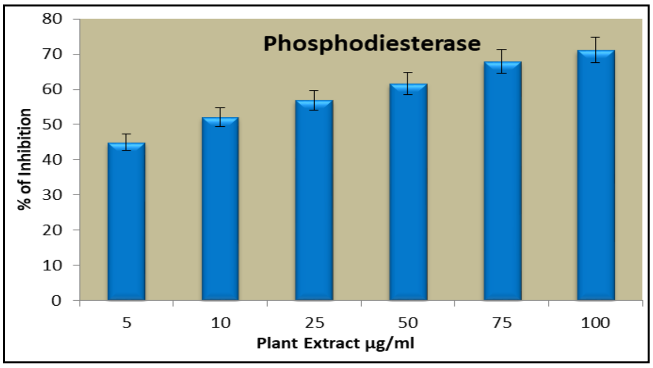

Fig 4: Inhibition of Phosphodiesterase Activity

Conclusion

Aqueous extract from *Salvia leucantha* was shown to inhibit the *N.naja* venom enzymes hyaluronidase, acetylcholinesterase, 5'nucleotidase and phosphodiesterase in an *in vitro* model. Plant extracts could be alternative therapy for snake bite. These extracts provide satisfactory remedy for venom induced toxicities. Thus, it is important for the researchers, clinicians and importantly governing bodies to focus on the issues related to snakebite management in the subcontinent. Several studies on snake bite envinomation revealed that the medicinal plants have been used to treat snake bite, *in vitro* antivenom efficacy and other studies reveal that the current clinical management done using available antivenom as the treatment for the envenomation due to cobra bites in India. Further, *In vivo* model and other biochemical studies should be conducted to determine the intraregional variation in the venom composition and their exact mode of action on different organs.

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Conflict of Interest
The author(s) declare that they have no conflict of interest.

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