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In Vitro Evaluation of Anti-Venom Potential of *Nata Kushtadi Yoga* against Cobra Venom

Pattanaik Jina* and Chalakh Sonali

Department of Agadtantra, Mahatma Gandhi Ayurved College Hospital & Research Center, Wardha, Maharashtra, India

**Corresponding Author*

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Abstract: Snake Envenomation is a serious medical concern due to the neurotoxic and cytolytic effects, especially from the Indian cobra (*Naja naja*), which are caused by enzymes including phospholipase A2 (PLA2) and acetylcholinesterase (AChE). Traditional Ayurvedic treatments for snake bites include *Natakushtadi Yoga*, which is mentioned in classical books like Ashtanga Hridaya and Charaka Samhita. The purpose of this study was to evaluate *Natakushtadi Yoga's* anti-venom potential using *in vitro* enzymatic assays. Authentic *Nata* and *Kustha* were grounded into fine powders and made in accordance with traditional methods. For standardization, High Performance Thin Layer Chromatography (HPTLC) was employed to verify uniformity and quality of formulation. Using Ellman's technique to assess acetylcholinesterase inhibition, the formulation demonstrated a dose-dependent inhibitory effect, with an IC_{50} value of 310.5 µg/ml and a maximal inhibition of 82.59% at 500 µg/ml. Using an indirect hemolytic assay to measure phospholipase A2 inhibition, the venom-induced hemolytic halo decreased from 16 mm to 7 mm as formulation concentration increased. Furthermore, the venom-induced fibrinolytic activity was significantly inhibited by *Natakushtadi Yoga*, lowering the fibrinolytic halo to 6 mm at the maximum dosage. These results imply that by blocking important enzymatic processes of cobra venom, *Natakushtadi Yoga* has anti-venom qualities.

Keywords: Envenomation, Antidote, Anti-Venom, *Natakushtadi Yoga*, Cobra Venom

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Introduction

Venomous snakebites represent a severe medical, social, and economic challenge in many parts of the world, particularly in tropical and subtropical nations. Majority of the world's dangerous snakes are found in developing countries (Saini *et al.*, 1984; Kasturiratne *et al.*, 2008). Indian Cobra is one of the snakes commonly associated with human mortality in India. The cobra venom produces systemic poisoning because of rapid action of neurotoxins which causes respiratory

paralysis and death (Gaitonde and Bhattacharya, 1980; Achyuthan and Ramachandran; 1981; Morais and Massaldi, 2009). The most common enzymes involved in the patho-physiology of snake venom includes Phospholipase A2s (PLA2s), metalloproteinases, acetylcholin-esterases (AChEs), nucleotidases and hyaluroni-dases (Prody *et al.*, 1987; Dave *et al.*, 2000).

The antiserum is the main therapeutic option available for the treatment of snakebite; however, this does not provide enough protection against the severe symptoms of poisoning. Furthermore development of antiserum is very time consuming, expensive and requires ideal storage condition. To overcome these drawbacks, there is a need to develop new affordable and suitable antidote against snakebite. Currently, many plants and plant materials are being screened for their pharmacological activities as an anti-dote especially those used in traditional/folk medicine. Plant extracts, fractions and isolates have demonstrated the inhibitory activity against the snake venoms, these natural anti-dotes not only reduce the local tissue damage but also delay the diffusion of systemic toxins, therefore increases the survival time of the patient (Meenatchisundaram Dave *et al.*, 2009; Makhija and Khamar, 2010; Shinwari; 2010).

Nata (*Valeriana wallichii*) also known as *Tagara* in Ayurveda is a hairy perennial herb belongs to Valerianeaceae family, growing in the temperate regions of the Himalayas and other hills area. It has been used in Ayurveda as a medicine for various ailments and disorders from centuries. The root of this herb contains Valerinic acid and Valepotriates which have been used as sedative and tranquilizers. Essential oils were also obtained from the root and dried rhizomes. The essential oil contains valeric acid, sesquiterpene, camphene, terpene alcohol and terpineol (Hansel *et al.*, 1982; Sharma, 2001).

Kustha is well known for its therapeutic properties, used for the treatment of fevers, skin diseases and headache, etc. Almost all Nighantus carry the description of *Kustha* with several synonyms. Some scholars consider two varieties of *Kustha* i.e. sweetish and bitter, but one with bitter taste is considered as real *Kustha*. In Ayurveda the root is used particularly for improving complexion, cures itching, ringworm, scabies and epilepsy. It is also used for treating paralysis, ophthalmic condition and headache, etc. (Kritikar and Basu, 1987; Ahmad and Javed, 2007; Madhuri *et al.*, 2012).

The aim of the present study was - (i) to prepare and standardize *Natakushtadi yoga* by analytical study; and (ii) to perform assessment of *Natakushtadi Yoga* against cobra venom for anti-venom enzymatic activities-- (a) to evaluate Acetylcholinesterase inhibition activity of *Natakushtadi yoga* against cobra venom; and (b) to evaluate Phospholipase A2 (PLA2) inhibition activity of *Natakushtadi yoga* against cobra venom.

Materials and Methods

Raw drugs; *Nata* and *Kustha* for the preparation of *Nata Kusthadi Yoga* were procured from reliable medicinal drugs provider. The raw drug material of *Nata* and *Kustha* were identified and authenticated from *Dravya Guna* Department of Mahatma Gandhi Ayurved College, Hospital and Research Centre Salod (H), Wardha (Maharashtra) India.

Pharmaceutical Processing

Preparation of Nata churna

After authentication of raw *Nata* roots it was used for further pharmaceutical preparation. Dry *Nata* root was collected and dried in shade for 1-2 days; *Nata* root was grinded to get powdered form of raw material, and then passed from Sieve No. 85. This finely powdered Churna was stored in air tight jar.

Preparation of Kustha churna

Dry *Kustha* root was collected, dried in shade for 1-2 days. After authentication of raw *Kustha* root it was used for further pharmaceutical preparation. *Kustha* root was grinded and pulverized to powdered, passed from Sieve No. 85. This Churna was then stored in air tight jar.

Preparation of Natakusthadi Yoga

Drug was prepared in Pharmacy of Mahatma Gandhi Ayurved College, Hospital andResearch Centre Salod (H), Wardha as per the reference of Ch. Chikitsa 23/194. All the individual ingredients which were authenticated, dried in shade, powdered, and sieved separately were subjected for process of mixing. Required quantities of drugs (powder) were weighed separately. Then all the powders were mixed uniformly by the process of spatulation in a vessel. The mixed formulation was unloaded and weighed; then it was stored in air tight container (Shastry, 2004; Sharma, 2006).

Standardization by instrumental technique (HPTLC)

For the HPTLC study following samples were prepared:

Sample – Id-1: *Nata*

Sample – Id-2: *Kustha*

Sample – Id-3: *Natakusthadi Yoga*

Sample preparation method

Sample was taken in flask and 50 ml 10% methanol was poured in it and shaken in magnetic stirrer for 6 h and kept overnight. Next day the mixture was filtered rapidly and only supernatant was collected and used for further study.

Chromatographic Conditions:

Development Chamber:	Camag Twin Trough Chamber (10x10 cm^2)
Application Mode:	Hamilton Syringe (100 ml), CamagLinomat 5
Plates:	HPTLC Silica gel 60 F254
Application Rosetin :	10 mm
Chamber Saturation:	30 min
Detection Saturation:	Camag TLC scanner
Lamp:	Deuterium lamp, mercury lamp
Data System:	WinCats software
Sample:	Standard
Mobile Phase:	Toulene: Ethyl Acetate 7:3

Stationary Phase

Material:	HPTLC plates silica gel 60 F 254
Plate size (X x Y):	10.0 x 10.0 cm
Manufacturer:	E. MERCK KGaA

Pharmacological Study

Preparation of Snake venom:

The Lyophilized Indian cobra venom was obtained and preserved at 4°C. Before use, the venom was dissolved in normal physiological saline (0.9%) and centrifuged at 3000 rpm for 10 min and the supernatant was used for anti-venom studies.

Preparation of Extract of Natakushtadi Yoga (Stock solution)

The *Natakushtadi Yoga* was allowed to dry in shade. Then macerated with 100 ml distilled water and allowed to stand for 24 h. Thereafter the solution was filtered and evaporated to dryness under reduced pressure at 40°C. This stock solution was further utilized to prepare different dilutions.

Acetylcholinesterase Inhibition Activity

Acetylcholinesterase inhibitory activity was assayed by following Ellman *et al.* (1961) method (Ingkaninan *et al.*, 2006). The reaction mixture comprised 3.0 ml of the phosphate buffer (pH 8.0), 10 µl of 5,5'-Dithiobis-2-Nitrobenzoic Acid (DTNB) and 20 µl of acetylethiocholine iodide (158.5 mmol/l). A total of 50 µl of 0.1% crude venom and 3 ml of buffer solution were incubated at room temperature for 5 min. Then, 10 µl of DTNB and 20 µl of substrate acetylthiocholine iodide were added in order to reach a final concentration of 1 mmole/l. The increase in absorbance at 412 nm was measured on a double beam spectro-photometer against control mixture prepared at the same time. However, in the later case, 50 µl of enzyme was replaced with 50 µl of buffer solution. For the inhibition studies, venom was pre-

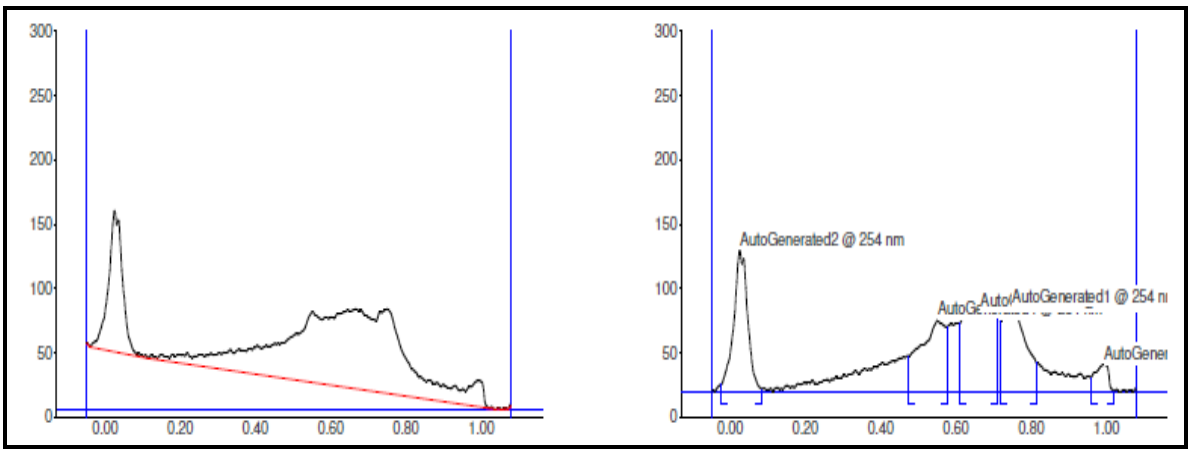

Fig. 1: HPTLC results of *Nata* at a wavelength of 254 nm.

incubated with extract (test) or standard mixture for 30 min at 37°C. Following mixtures were served as control, standard and test solutions for this study:

Control Solution: 3 ml phosphate buffer + 10 µl DTNB + 20 µl Ach iodide + 50µl venom + 50 µl buffer solution.

Standard Solution: 3 ml phosphate buffer + 10 µl DTNB + 20 µl Ach iodide + 50 µl venom + 50 µl enzyme solution.

Test Solution: 3 ml phosphate buffer + 10 µl DTNB + 20 µl Ach iodide + 50 µl venom + 50 µl formulation (test compound).

Phospholipase A2 Inhibition Activity

Phospholipase A2 activity was measured using an indirect hemolytic assay on Agarose-egg yolk gel plate (Dhananjaya *et al.*, 2011). Increasing doses of cobra venom were added to 3 mm wells in agarose gels (0.8% in PBS, pH 8.1) containing 1.2% sheep erythrocytes, 1.2% egg yolk as a source of lecithin and 10 mM $CaCl_2$. Plates were incubated at 37ºC overnight and the diameters of the hemolytic halos were measured. The minimum indirect hemolytic dose (MIHD) corresponds to a dosage of venom, which produced a hemolytic halo of 11 mm diameter. The efficacy of plant extracts in neutralizing the phospholipase activity was determined by mixing constant amount of venom with various amount of sample (0.6, 0.8, 1.0, 1.2 and 1.4 mg/ml) and incubated at 37ºC for 30 min. Then aliquots of 10 µl of mixtures were added to wells in agarose egg yolk sheep erythrocyte gels. Plates were incubated at 37ºC for 20 h. Neutralization was expressed as concentration of plant extract which could reduce the hemolytic halo by 50% when compared to the effect induced by venom alone.

Results and Discussion

High Performance Thin Layer Chromatography (HPTLC) analysis of *Natakusthadi Yoga* was performed using a mobile phase composed of Toluene and Ethyl Acetate in a 7:3 ratio. The R_f value observed for *Natakusthadi* was 0.63, indicating a distinct separation on the chromatographic plate. Figure 1 displays the HPTLC results of *Nata* at a wavelength of 254 nm, while Figure 2 represents the results for *Kustha* at the same wavelength. Figure 3 illustrates the HPTLC results of the combined formulation, *Natakusthadi Yoga*. Figure 4 shows a comparative chromatographic representation of all tracks at 254 nm, highlighting the MSDC (multi-sample detection chromatogram) along with its individual ingredients.

The HPTLC analysis of *Nata*, *Kustha*, and their polyherbal formulation *Natakusthadi Yoga* was

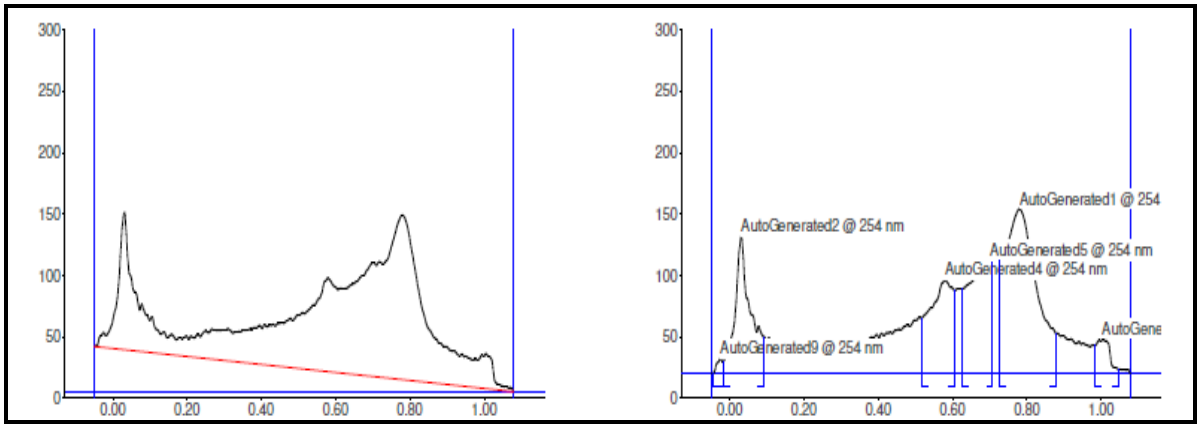

Fig. 2: HPTLC results of *Kustha* at a wavelength of 254 nm.

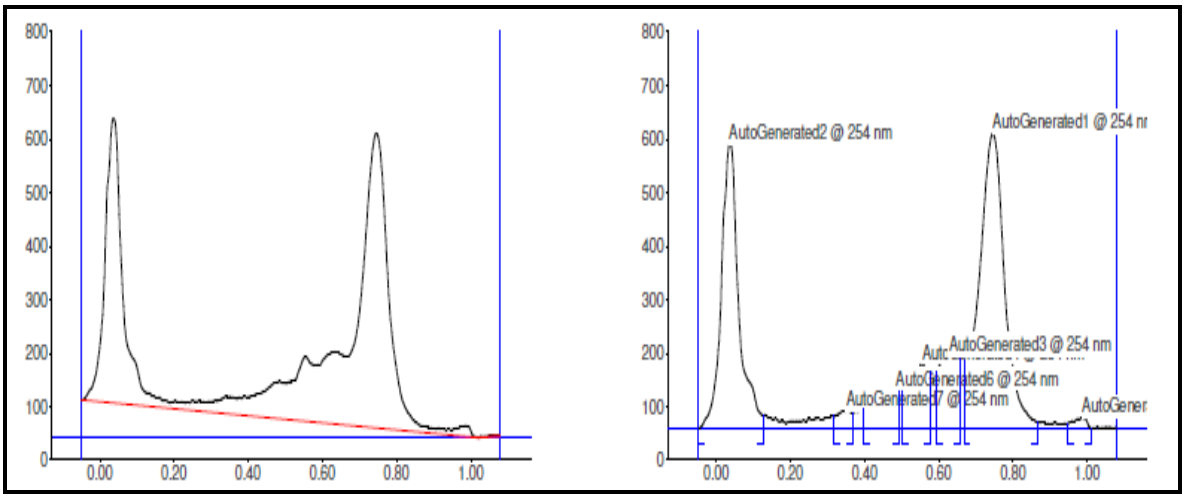

Fig. 3: HPTLC results of *Natakusthadi Yoga* at a wavelength of 254 nm.

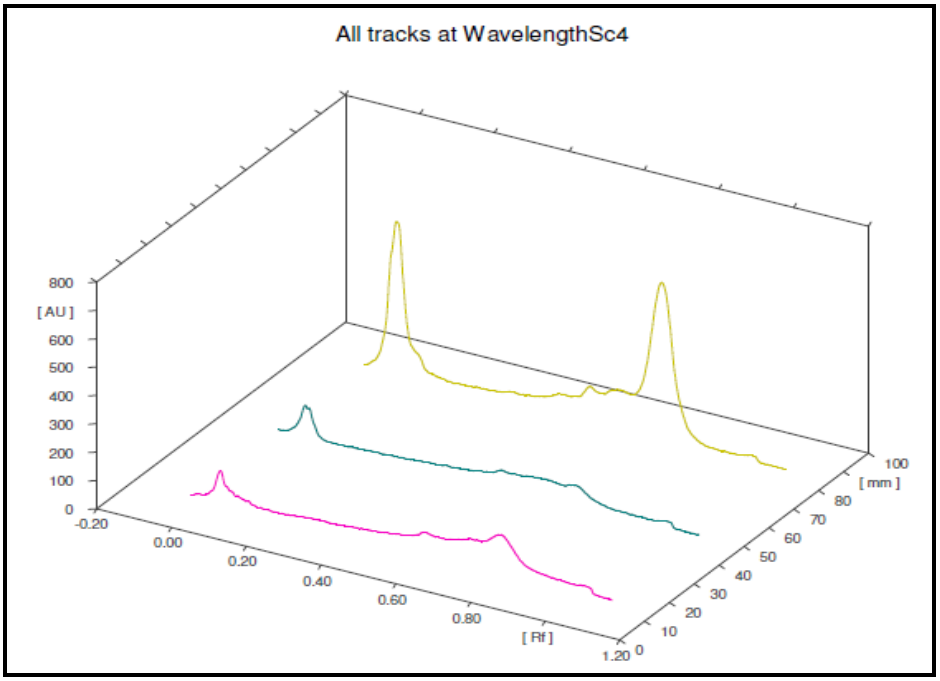

Fig. 4: Comparative chromatographic representation of all tracks at 254 nm.

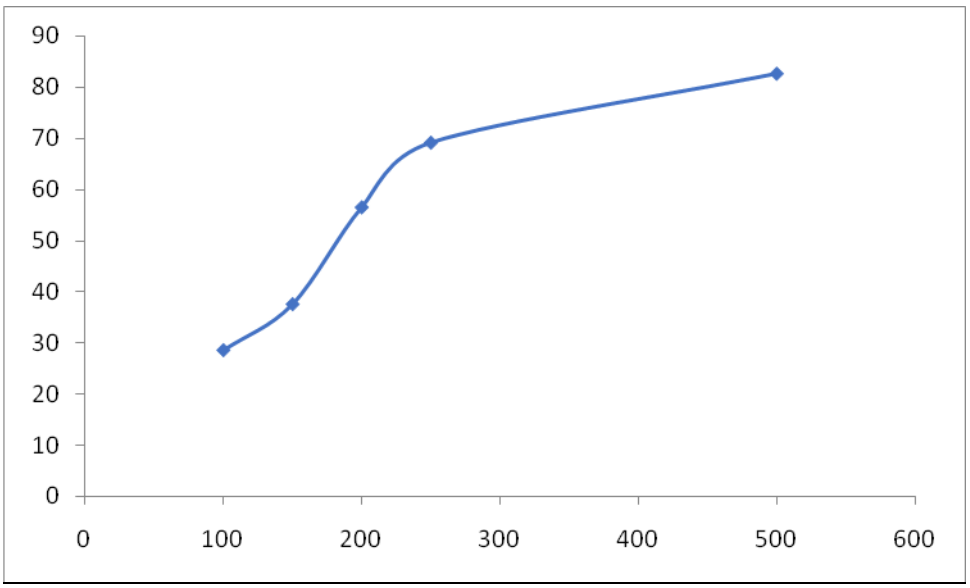

Fig. 5: % Inhibition of acetylcholinesterase activity by the *Natakushtadi yoga.*

performed at a wavelength of 254 nm to evaluate their phyto-chemical profiles and establish a comparative fingerprint. Figure 1 shows the chromatogram of *Nata*, which reveals distinct peaks at specific Rf values, indicating the presence of characteristic phytoconstituents. Figure 2 presents the chromatogram of *Kustha*, showing multiple peaks that differ in number and position from those in *Nata*, suggesting a unique chemical composition. Some overlapping Rf values indicate the presence of common constituents. Figure 3 illustrates the chromatogram of *Natakusthadi Yoga*, displaying a more complex pattern with peaks corresponding to both *Nata* and *Kustha*, along with additional peaks that may result from synergistic interactions or chemical changes during formulation. Figure 4 provides a comparative representation of all three tracks on a single plate, highlighting the similarities and differences in their chemical profiles. The presence of shared peaks confirms the incorporation of individual components, while the unique peaks in the compound formulation suggested the formation of new bioactive compounds. This comprehensive chromatographic evaluation confirms the successful formulation of *Natakusthadi Yoga* and offers a reliable fingerprint for future quality control.

Inhibition of acetylcholinesterase activity by the Natakushtadi Yoga

The *Natakushtadi yoga* was found to be effective in the inhibition of acetylcholinesterase activity. The maximum inhibition (82.59%) was found at concentration of 500 µg/ml. The formulation inhibited the enzyme acetylcholinesterase in a dose dependent manner (Fig. 5). The IC_{50} value of different concentration of plant extract was found to be 310.5 µg/ml (Table 1).

These data suggested that *Natakushtadi yoga* may have the potential to inhibit the activity of acetylcholinesterase (AChE), an enzyme involved in the nervous system. The percentage inhibition of AChE activity increases with increasing concentrations of *Natakushtadi yoga*.

Phospholipase A_2 inhibition activity

Indirect hemolysis assay:

The indirect hemolysis assay was performed to determine the inhibition of phospholipase A_2 by the *Natakushtadi yoga*. The Minimum Indirect Hemolytic Dose (MIHD) (200µg/ml) of snake venom that produced hemolytic halo of 16 mm diameter was determined. This hemolytic zone

Table 1: % Inhibition of acetylcholinesterase activity by the *Natakushtadi yoga*

S. No.	Concentration (µg/ml)	% Inhibition (mean ±SEM)	IC_{50} (µg/ml)
1	100	28.56±0.18	310.5
2	150	37.56±0.51	
3	200	56.48±0.26*	
4	250	69.11±0.12*	
5	500	82.59±0.31**	

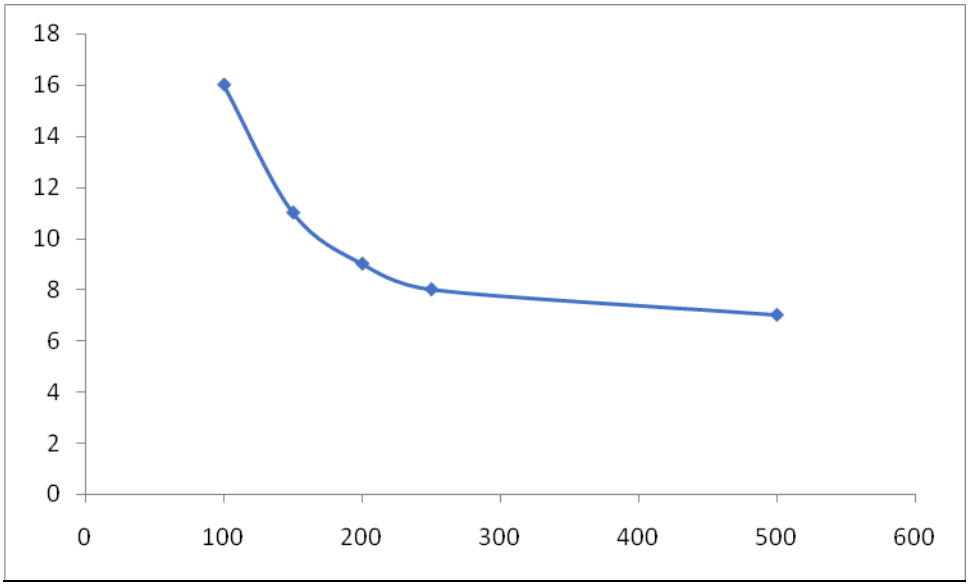

Fig. 6: Reduction of venom induced hemolytic zone by *Natakushtadi yoga.*

Table 2: Effect of *Natakushtadi Yoga* in reducing hemolytic halo produced by venom

S. No.	Venom dose to produce 11 mm zone	Concentration of drug (µg/ml)	Reduction in zone by drug (mm)
1	250 µg	100	16
2		150	11
3		200	9
4		250	8
5		500	7

produced by the snake venom was reduced by the *Natakushtadi yoga* in a dose dependent manner (Fig. 6). The maximum reduction of hemolytic halo was observed at the dose of 500 µg/ml of *Natakushtadi yoga* (Table 2).

Natakushtadi yoga also showed potential to inhibit the activity of PLA_2, an enzyme found in snake venom and involved in inflammatory processes. The method utilizes red blood cells (RBCs) to indirectly evaluate the inhibition of phospholipase A2 (PLA2) activity. Snake venom containing PLA2 enzymes causes lysis of RBC membranes, leading to the formation of a clear hemolytic zone (halo) around the site of venom application. When *Natakusthadi Yoga* was introduced, it potentially inhibits PLA2 activity, thereby reducing the venom-induced hemolysis. The experiment demonstrated that as the concentration of *Natakusthadi Yoga* increased, the size of the hemolytic zone decreased from 16 mm

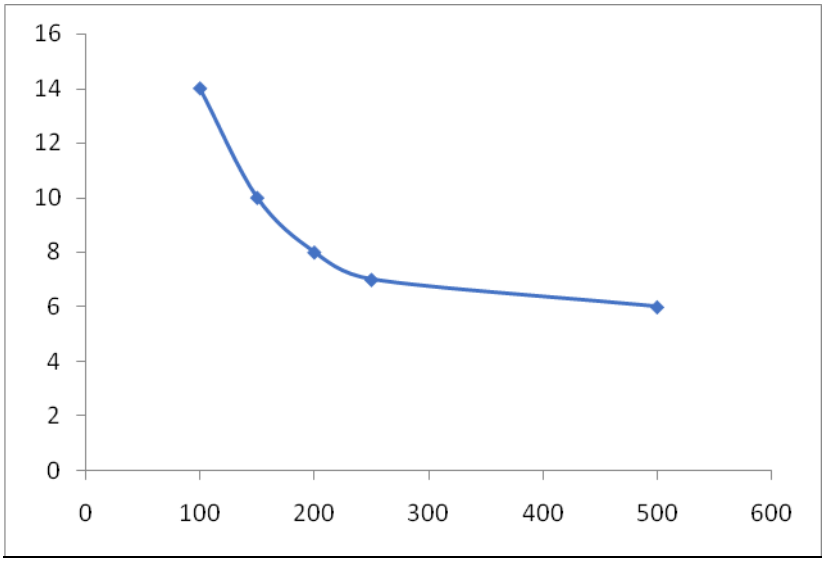

Fig. 7: Reduction in venom induced fibrinolytic halo by *Natakushtadi yoga.*

Table 3: Reduction in venom induced fibrinolytic halo by *Natakushtadi yoga*

S. No.	Venom dose to produce 11mm zone	Concentration of drug (µg/ml)	Reduction in Fibrinolytic halo by drug (mm)
1	250 µg	100	14
2		150	10
3		200	8
4		250	7
5		500	6

to 7 mm; indicating a dose-dependent inhibition of PLA2.

Inhibition of Fibrinolytic Activity

In inhibition of fibrinolytic activity the *Natakushtadi yoga* showed effective reduction of fibrinolytic halo produced by the snake venom (Fig. 7). The Minimum Fibrinolytic Concentration (MFC) produced by the snake venom was found to be 200 µg/ml. The maximum reduction of fibrinolytic halo was observed in the dose of 500 µg/ml of *Natakushtadi yoga* (Table 3).

Conclusion

Snakebite continues to pose a significant public health challenge, especially in rural and tropical regions, where access to anti-venom and medical care is limited. Despite advancements in modern medicine, many affected individuals still rely on traditional remedies, including medicinal plants, for the treatment of snake envenomation. Historically, herbal medicines have played a vital role in health care systems across the world, with many communities using plant-based formulations to neutralize snake venom. The present study focuses on *Natakusthadi Yoga*, a classical polyherbal formulation, and its potential anti-venom activity against cobra (*Naja naja*) venom. The venom of *Naja naja* is known to contain a complex mixture of neurotoxins, cardiotoxins, and enzymes such as acetylcholin-esterase and phospholipase A2 (PLA2), which disrupt neuromuscular function and cause systemic toxicity. The study demonstrated that *Natakusthadi Yoga* exhibited significant acetylcholinesterase inhibition, which may delay the binding of venom neurotoxins to acetylcholine receptors, thereby potentially reducing the risk of

respiratory paralysis. Additionally, it showed PLA_2 inhibition, which helps to prevent hemolysis and damage to red blood cells, along with fibrinolytic inhibition, suggesting its role in maintaining blood integrity and preventing rapid clotting triggered by venom components. The drug also exhibited dose-dependent enzyme inhibition, suggesting the presence of active phytoconstituents capable of neutralizing venom effects. These findings support the traditional claims of *Natakusthadi Yoga* as a potential antidote and advocate for its further validation through *in vivo* studies, phytochemical isolation, and pharmacological assessments. *Natakusthadi Yoga* and similar formulations may serve as valuable adjuncts or alternatives in snakebite management, especially in emergency scenarios and post-bite care.

Ethical Statement

Study was commenced in accordance with the SOP and ethical guidelines prescribed by Good Laboratory Practices (Approval for same was received from "Institutional Ethics Committee" Ref. No. MGACHRC/IEC/August-2020/108, Dated-21.08.2020).

Author Contributions

Each author has contributed equally in designing the research, conducting thorough analysis, drafting the manuscript, and refining its content. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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