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"Evaluation of antivenom potential of NataKushtadi Yoga against cobra venom, in-vitro study''

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Abstract

Background: The Indian Cobra (Najanaja) is considered very dangerous snake and it is commonly associated with high human mortality rate in India. Due to rapid action of neurotoxins it produces systemic poisoning which causes respiratory paralysis, the major cause of death. Cardio respiratory support and Anti Snake Venom (ASV) are the most important and effective tools for snake bite treatment. ASV has many side effects like anaphylaxis,pyrogen reactions and serum sickness.To avoid the above stated risk due to ASV treatment there is anurgent need to develop and search the affordable and suitable antidote as alternative treatment. The Indian Medicinal Plants extracts has veryplentiful source of pharmacologically active compounds and theirextracts has property to act against snake venom.The present study has been planned with objective to examine the therapeutic potential of Natkusthadi yoga by analytical study and as an antidote to neutralize the adverse action of snake venom by acetylcholinesterase and Phospholipase A2 (PLA2)inhibition activity.

Aim:- Assessment of Natakushtadi Yoga for anti-venom enzymatic activities against cobra venom.

Material &method:-The Acetylcholinesteraseinhibition activity will be measured by following Ellman et al. method and Phospholipase A2 (PLA2)inhibition activity will be measure using an indirect hemolytic assay on Agarose-egg yolk gel plate by following BrindhaDurairaj Method.

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Result: - The water extract of Natakushtadi Yogawill manifest a notable inhibitory effect on Acetylcholinesterase and Phospholipase A2 (PLA2) enzymes found in cobra venom.

Keywords:- Acetylcholinesterase, Phospholipase A2 (PLA2), Natkusthadi yoga, cobra venom.

INTRODUCTION (Background/ objective)

World Health Organization (WHO) documents thatmost of the world Semi- urban &Ruralpopulation has dependency on traditional or herbal medicine for fulfil their primary health care needs. In traditional system of medicine the plants based medicines and products have been used worldwide to cure different type of diseases[1]. For the development of snake venom antagonists, many attempts have been made over the years from several plant sources despite of the existence of antiserum[2-3].
The Indian Cobra (Najanaja) is considered very dangerous snake and it is commonly associated with high human mortality rate in India. Due to rapid action of neurotoxins it produces systemic poisoning which causes respiratory paralysis, the major cause of death [4-6].

There are more than hundreds types of different proteins& enzymesfound invenom. Viperid venom constitute 80-90% enzymes and elapid venoms contains 25-70% enzymes, nerve growth factor (non-toxic proteins) and non-enzymatic polypeptide toxins. It contains most powerful neurotoxins which act on postsynaptic junction and lowin molecular weight and diffuses rapidly through blood stream.

A large amount of acetylcholinesterase (AChE) enzyme found in Elapidaefamilywhichcauses the inactivation of acetylcholine (physiological events controller) by the enzymatic intervention resulted into respiratory failure by blocking diaphragm muscle[7-8].

Phospholipase A2 (PLA2) enzyme found in snake venom causes hemolysis of RBCs by acting on Human RBCs (HRBC) membrane associated with phospholipids liberating lysolecithin. Due to manifestation of injury inRBC membrane, the RBCs cellsbecome more susceptible to secondary damage through free radicals.

The only effective treatment available for snake bite areCardio respiratory support and Anti Snake Venom (ASV). Anti SnakeVenomhasmany side effects like anaphylaxis, pyrogen reactions and serum sickness which causes adverse effect on the body. To avoid the above stated risk due to ASV treatment there is an urgent need to develop and search the affordable and suitable antidote as alternative treatment.Many medicinal plants have potential toincrease the snakebite victim survival time, enhance diaphragm muscle contraction, decrease the severity of toxic signs, inhibit protein destruction andblock antibody attachment to venom,

In ayurvedicclassical textsthe snakes (Sarp) are broadly classified into five groups A) *Darvikara*(hooded type), B) Mandali (hoodless and skin is painted with varied colors of circular paths or rings), C) Rajimanta (striped and hoodless), D) *Nirvisha*(non-poisonous snakes) and E) *Vaikaranja*(hybrid species).The signs and symptoms of these snakebites expressed in each *Vega* (stage) indicatesthe spread of the poison from one tissue to the other

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and it is also therapeutically important because the management depends on the stage at which the poison has spread in the body.

To counter- act the action of sarpvisha, ancient Acharyas have mentioned about the Agada'sor vishnashak yoga's . These Agadasor vishnashak yoga's are anti- poisonous remedies which are used in various types of snakebite conditions . Some Agador vishnashak yoga's are target specific which act on particular venom or poision.

Natakusthadiyoga is one of the herbal combination mentioned in CharakSamhita ,Chikitsasthan, Vishchikitsa (23/ 194) &AstangHridya, Uttarsthan, SarpvishpratishedAdhyaya 36/73 , used for treatment of snake bite leading to severe risk of life. Natakusthadi yoga is prepared by the equal quantity (1pala each) of powder of Nata(ValerianaWallichii DC) and Kustha (SaussureaLappa C.B. Clarke) added with Ghee and Madhu (2 pala each) and consumed internally to destroy the TakshaksarpVisha. In this herbal formulation, Nata&Kustha are two major contents.

Nata&Kushta medicinal plants are easily available in Indian diaspora and both are widely used in many Agada mentioned in classical texts. Natakushtadi Yoga is easily prepared and cost effective. Both have reported acetylcholinesterase and PhospholipaseA2 enzyme inhibiting property in many Research studies.

Table No. 1 – References of Nata and Kusthamentioned in different Ayurvedic texts.

S.No.	**Nata(ValerianaWallichii DC)**		**Kustha (SaussureaLappa C.B. Clarke)**	
1	Masyadi Yoga	C. Chi.23/ 190	Chandnadi yoga	C.Chi. 23/ 192
2	Chandnadi yoga	C.Chi. 23/ 192	TakshryaAgada	Su. K. 5/ 65
3	Vyoshadi Yoga	Ch. Chi. 23/197-198	SarvkarmikAgada	Su. K. 5/ 78-80
4	Kutajadi Yoga	Ch. Chi. 23/206-207	RishabhAgada	Su. K. 5/ 68-72
5	AjitMahagada	Su. K. 5/ 64	NatadiAgada	A.H.U.36/ 73
6	TakshryaAgada	Su. K. 5/ 65	KatukadiAgada	A.H.U.36/67
7	SarvkarmikAgada	Su. K. 5/ 78-80		
8	NatadiAgada	A.H.U.36/ 73		
9	VajraAgada	A.H.U. 36/ 82&83		
10	BilwadiAgada	A.H.U. 36/ 84&85		

Thus, the present study has been planned with objective to examine the therapeutic potential of Natkusthadi yoga as an antidote to neutralize the adverse action of snake venom by acetylcholinesterase and Phospholipase A2 (PLA2)inhibition activity.

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MATERIAL AND METHOD

Natakushtadi Yoga (Test formulation), Lyophilized Cobra Venom, DTNB (a strong oxidizing agent), Acetylthiocholine iodide, Phosphate buffer, Agarose, Egg yolk, Sheep erythrocyte, Calcium chloride, double beam spectrophotometer etc will be used in the proposed study.

A- Analytical Study

The plant material required for preparation of Natakushtadi Yoga will be collected from different areas. Raw drugs will be verified and authenticated by Dravyaguna department. Natakusthadi yoga is prepared by the equal quantity (1pala each) of powder of Nata(ValerianaWallichii DC) and Kustha (SaussureaLappa C.B. Clarke) dried rhizomes. Organoleptic and physicochemical properties of Natakusthadi yoga will be carried out.These studies and HPLC fingerprint profiles will be useful for deciding the identity, purity and strength of the Natakusthadi yoga.

B- Experimental Study

The Lyophilized Indian cobra venom is obtained from Parassinikadavu Snake Park & Zoo, Parassinikadavu, Kannur, Kerala and it will be preserved at 4∘C. The venom will be dissolved in normal physiological saline (0.9%) before its use, and centrifuge for 10min at 3000 rpm and the supernatant will be used for antivenom studies.

Extract of Natakushtadi Yoga (Stock solution)

The Natakushtadi Yoga will allow to dry in shade. Then 5 gm dried yoga is macerated with 100 ml distilled water and allow to stand for twenty-four hours then filtrate will be used for further study.

Different dilutions of Stock solution

5 dilutions of different concentration will be prepared from stock solution. The concentration of solutions that inhibit the hydrolysis of substrate by 50% (IC 50) will be determined by evaluating and monitoringthe effect of various concentrations.

Determination of IC50

It is a quantitative measure;the IC 50 value is that concentration of a drug that reduces the activity of another drug to a biological component (an enzyme, cell, cell receptor or microorganism) by 50%.

To calculate IC50 first we will take absorbance of all the dilutions.

1. Make a scatter graph in excel (where X axis is concentration and Y axis is % activity)
2. Get the slop equation (Y = m x + c or Y = m x - c) for the graph.
3. For IC50 value in equation

Y=50

M and C values will be present in the equation itself.Then find out the value of “X”

4. Value of X will be IC50 value for that graph.

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Acetylcholinesterase inhibitory Activity

Acetylcholinesterase inhibitoryactivity will be measured by following Ellman et al. method[9-10].The reaction mixture will be made by10 µL of DTNB (10 mmole/L), 3.0 mL of the phosphate buffer (pH 8.0), and 20 µL of acetylethiocholine iodide (158.5 mmol/L). At room temperature 50 µL of 0.1% crude venom and 3 mL of buffer solution will be incubated for five minutes. Then, 20 µL of substrate acetylthiocholine iodide and 10 µL of DTNB (a strong oxidizing agent) will be added in order to reach a final concentration of 1 mmole/L. Thenwith the help of double beam spectrophotometer an increase in absorbance will be measured at 412 nm against control mixture prepared at the same time. However, 50 µL of enzyme will be replaced with 50 µL of buffer solution in the later case. For the inhibition studies, venom is preincubate with extracts/Test or standard mixture for 30 minutes at 37°C.

Three mixtures will be prepared :-

- 3 ml phosphate buffer + 10 µl DTNB + 20 µl Ach iodide + 50 µl venom + 50 µl Buffer solution – **Control mixture**
- 3 ml phosphate buffer + 10 µl DTNB + 20 µl Ach iodide + 50 µl venom + 50 µl enzyme – **Standard mixture**
- 3 ml phosphate buffer + 10 µl DTNB + 20 µl Ach iodide + 50 µl venom + 50 µl Test formulation– **Test mixture**

Then, % inhibition of Standard and Test mixture will be obtained by following calculation after measuring the absorbance on a double beam spectrophotometer at 412 nm.

- % inhibition of Standard mixture = [(control mixture Absorbance – (Absorbance of standardmixture) /(control mixture Absorbance)] x 100

- % inhibition of Test formulation = [(control mixture Absorbance – (test formulation Absorbance)/(control mixture Absorbance)] x 100

Phospholipase A2 inhibitory activity

Phospholipase A2 inhibitory activity will be measured by using an indirect hemolytic assay on Agarose-egg yolk gel plate by following BrindhaDurairaj method[11]. Increasing doses of cobra venom will be 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 10mM CaCl2. Then, Plates will incubate at 37ºC overnight and the diameters of the hemolytic halos will be measured. The minimum indirect hemolytic dose (MIHD) corresponds to a dosage of venom, which produce a hemolytic halo of 11mm diameter. The efficacy of plant extracts in neutralizing the phospholipase activity will be determine by mixing constant amount of venom with various amount of sample (0.6, 0.8, 1.0, 1.2 and 1.4mg/ml) and incubate at 37ºC for 30 minutes. Then 10µl aliquots of mixtureswill be added to wells in agarose egg yolk sheep erythrocyte gelsand plateswill beincubated at 37ºC for 20 hours. Neutralization will be manifest as concentration of plant extract which would reduce the hemolytic halo by 50% when compared to the effect induced by venom alone.

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Mean ± standard deviation (SD) will be used for assessment of Data. All analyses will be planned in triplicates andone-way ANOVA will be used for statistical analyses. Groupsdifferences will be determined at $p < 0.05$.

Anticipated / expected results

The Sample of Natakushtadi Yoga will be subject to physicochemical analysis organoleptic analysis, and High performance liquid Chromatography (HPLC) examination by optimizing the solvent systems. Pharmacognostical profile of Natakushtadi Yoga will be established. Specific gravity, Loss on drying, Iodine value, Viscosity and Refractive index, Acid value and Saponification values ofNatakushtadi Yogawill be presumed withinprescribed limits.
Enzymatic inhibition study will be reveals that the water extract of Natakushtadi Yoga is able to inhibit acetylcholinesterase and Phospholipase A2 (PLA2) enzymes found in cobra venom.

DISCUSSION

The extracts of Indian Medicinal Plants has veryplentiful source of pharmacologically active compounds and these extracts has been shown to act against snake venom in various studies[12-14]. Many studies revealed that medicinal plants have potential to increase survival time in snake bite victims, decrease the severity of toxic signs, increased diaphragm muscle contraction, inhibit protein destruction and block antibody attachment to venom. Antivenom is available but its availability is limited and depends upon its development and standardization which are found to be very expensiveand difficult. Due to this many efforts are continuously being made to invent alternative treatment strategy from medicinal plants, which would be cost effective and free of any adverse reactions too.

Nata& Kushta[11], being easily available, they are widely mentioned in many antitoxic classical preparations. The preparation of Natakushtadi Yoga is easy and cost effective. Both have reported acetylcholinesterase and PhospholipaseA2 enzyme inhibiting property in many Research studies.Various anti- venom formulations have been mentioned in Ayurveda which are narrated as highly effective. Therefore similar studies can be done on other such formulations either as single formulation or on comparative basis to discover better anti venom agent. Further evaluation is required to identify and isolate the active constituents found in Natakushtadi Yoga for antivenom activity.

Conflict of Interest: None

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