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## *In vitro* antitoxin activity of aqueous extracts of selective medicinal herbs against *Naja naja* venom

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### Abstract

There is increasing global health problems related to snake bites and its complications in India. In the medical practice, the only therapy for treating snake bite victims is anti-snake venom serum, but it has huge acute and chronic including late adverse reactions in humans. Thus this study was aimed to analyze the potential of selective medicinal herbs against *Naja naja* toxin by *in vitro* methods. The aqueous extracts of fruit, root, seed, bark and leaf of *Emblia officinalis*, *Hemidesmus indicus*, *Tamarindus indicus*, *Mangifera indica* and *Vitex negundo* were prepared respectively. Various concentrations of the extracts were used to evaluate *in vitro* anti toxin activity by acetylcholinesterase, protease, direct hemolysis, phospholipase and procoagulant activities. The results indicate that the root and bark extracts of *H. indicus* and *M. indica* possesses significant bioactive compounds that can neutralize the toxins of *N. Naja*.

**Keywords:** Toxin, *Naja naja*, *in vitro* antitoxin activity, medicinal herbs

### Introduction

Snake bite is a neglected public health issue that are largely found in tropical and subtropical countries including India where venomous snakes are the most abundant. It is a common occupational hazard mainly found among farmers, plantation workers etc that increasing the morbidity and mortality and are not reported properly [1]. The most affected region in the world is south east Asia due to sleeping in floor and open style habitation and WHO listed snake bite as neglected tropical conditions in 2009 because it has all the features including affects the poor, low priority in research, causes disability and stigma [2].

There are nearly 3,200 snakes' species in the world [3], among them 216 are in India where 60 are considered as poisonous [4]. The common poisonous snakes found in India are comes under the families Elapidae (*Naja naja*, *Ophiphagus hannah*) and Viperidae (*Daboia russelli*, *Trimeresurus gramineus*). India is reported with the highest number of snake bites (81,000) and mortality (11,000) per year and has the worst snakebite problem in the world. Severe health related disabilities are recorded in the form of paralysis, disfigurement and disability.

To overcome this, an urgent priority is required to make matters dire, effective, affordable and quality anti-venoms in India. Many of the anti-venom products in Indian pharmaceutical market are in poor quality and are also not potent enough and required more products for reaching adequate potency to have a therapeutic value. However, the snake venom form viper of Tamilnadu is different from a Viper's venom in West Bengal. To overcome this, the collection of snakes from the whole country and prepare the common antidote is required but the quality assurance is absolutely critical. Another major hurdle to overcome is there are no venom standards available with WHO itself. The exploration of herbal drugs for snake bite therapy is now emerging due to the large availability of the herbs, effective against toxins, low cost and less adverse effects. Some studies highlighted the herbs including *Pimpinella anisum* [5], *Salix alba* [6], *Dendroaspis polylepis* [7], *Azadirachta indica* [8], *Harpalyce brasiliiana* [9], *Glycyrrhiza glabra* [10], *Baccharis trimera* n [11], *Andrographis paniculata* [12] and *Cyclea peltata* [13] composed of phytoconstituents like anisic acid, salicylic acid, atropine, aiplai, edunol, glycyrrhizin, neo-clerodane, ursolic acid and absinthin respectively having wide anti-venom activity. In this study, we are concentrating on the snake venom of *Naja naja*, that are reported as a potential neurotoxin affects normally the central nervous system of humans leads to sudden death, paralysis and severe central nervous disorders. In order to reduce the mortality, morbidity and disabilities due to snake bite, the usage of medicinal herbs as anti-toxin drug for snake venom, this study is carried out to determine and explore medicinal herbs that are having active principle to act against snake venom toxicity by *in vitro* analysis.

## Materials and Methods

### Snake venom

The lyophilized powder of venom of *Naja naja* was obtained commercially (Sigma-Aldrich) which is whitish yellow in colour and it was stored at -80 °C until use. Stock solution was prepared by dissolving 1mg of lyophilized venom in 1ml of physiological saline (0.9% sodium chloride).

### Collection of herbs

Five different herbs that are medicinally important are included in this study. All the herbal explants were collected locally from Kancheepuram district of Tamilnadu (India). The medicinal importance and usage for anti-snake bite of the test herbs were collected from local experienced and elderly peoples and practicing vaidyars. The plant was botanically authenticated by the regional centre. The herbal plants and their explants included in this study are depicted in table 1.

**Table 1:** Description of the herbs included in this study

Herb	Parts used	Place of collection	Volume of collection	Reference (anti-venom)
<i>Emblica officinalis</i>	Fruit	Lathur	250 gms	14
<i>Hemidesmus indicus</i>	Root	Maduranthagam	100 gms	15
<i>Mangifera indica</i>	Stem bark	Chithampur	100 gms	16
<i>Tamarindus indicus</i>	Seed	Maduranthagam	250 gms	17
<i>Vitex negundo</i>	Leaf	Sriperumbadur	250 gms	14

The exact herbal explants were collected in a clean container, washed thoroughly to remove the external debris. The cleaned plant parts were cut into small parts and show dried completely. Then all are grinded into coarse powder for subjecting to crude extract preparation.

### Preparation of extract

The crude bioactive compounds of the coarse powder of herbal explants were extracted by soaking 20 grams of each powder in 180ml of sterile distilled water. This mixture was blended completely using glass rod for 10 minutes and kept at room temperature for 18 hours in aseptic condition. Then the extracts were filtered separately in Whatman No.1 filter paper and the filtrates were vaped at 50 °C until completely dried.

### In vitro anti-toxin activities

#### Acetyl cholinesterase activity [18]

Different dilutions of plant extract from 100 to 250µg were prepared. Along with these diluted plant extracts, 200µg of venom was added in the concentration of 1mg/ml. This mixture was incubated for 1 hour at 37 °C. On other hand, assay mixture was prepared (100µl of 75mM acetylcholine iodide in 1ml of phosphate buffer) and the final pH should be in 6.8. After incubation of the plant – venom mixture, the supernatant was added to the assay mixture and incubated for 15 minutes at room temperature in dark condition. The control of venom and assay mixture without plant extract was maintained. The absorbance at 412nm was measured and the acetylcholinesterase inhibition was calculated using the formula

$$\text{Inhibition (\%)} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

#### Proteolytic activity [19]

In this method, casein is used as a substrate where the test mixture was prepared by adding casein in 0.02m Tris-HCl buffer at the final pH of 8.5. Different dilutions of plant extract from 100 to 250µg were prepared. Along with these diluted plant extracts, 200µg of venom was added in the concentration of 1mg/ml. This mixture was incubated for 1 hour at 37 °C. Plant extract – venom mixture was blended with undigested casein and this undigested casein was precipitated by adding 1.5ml of 0.4M trichloroacetic acid and

centrifuged. Using Folin-Ciocalteu's reagent, the digested casein was determined. The control of venom and assay mixture without plant extract was maintained. The absorbance at 412nm was measured and the acetylcholinesterase inhibition was calculated using the formula

$$\text{Inhibition (\%)} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

#### Direct hemolysis assay [13]

By using RBC, *in vitro* haemolytic action of snake venom and plant extracts were studied. The citrated blood of 5ml was centrifuged at 2000rpm for 10 minutes, where pellets were washed twice with physiological saline; 0.5ml of RBC mixture and 5ml of saline served as a control. Different dilutions of plant extract from 100 to 250µg were prepared. Along with these diluted plant extracts, 200µg of venom was added in the concentration of 1mg/ml and 0.5ml of washed RBC served as experimental sample. The optical density of experimental and control samples were measured at 540nm and the hemolysis was calculated as

$$\text{Hemolysis} = \frac{\text{Experimental sample} - \text{control sample}}{100\% \text{ hemolysis}} \times 100$$

#### Indirect hemolysis assay

This is a method of determining the phospholipase activity on agarose-erythrocyte-egg yolk gel plate [20]. The plate media was prepared by mixing the 0.8% agarose, 1.2% sheep erythrocytes, 1.2% egg yolk and 10mM CaCl<sub>2</sub> in 0.8% PBS; final pH is adjusted to 8.1 and the mixture was poured in sterile petridishes and allowed to solidify. A wells of 3mm size was made with equal distances both between the wells and wells and plate walls. In general the venom can produce haemolytic zone of 11mm diameter. Various concentrations of plant extracts and constant venom (µg) was mixed and incubated at 37 °C for 30 minutes. Aliquots of mixture were added to wells, where venom without plant extract was used as control. All the plates were incubated for 20 hours at 37 °C and the diameter of the haemolytic zone was measured.

**Procoagulant activity** <sup>[21]</sup>

Various concentrations of venom was dissolved in 100µl of PBS whose pH is adjusted to 7.2 was added to human citrated plasma and incubated at 37 °C. Coagulation time was recorded and the minimum coagulant dose was determined as the venom concentration which induced clotting of plasma within 60 seconds. Plasma incubated with PBS alone is used as control. For performing neutralization assay, venom was mixed with various concentrations of the plant extracts and the mixture was incubated for 30 minutes at 37 °C. After incubation, 0.1ml of mixture was added with 0.3ml of citrated plasma and the clotting times were recorded and the neutralization was expressed as effective dose (ED<sub>50</sub>). It was described as ratio µl of plant extract divided by mg of venom

used at which the clotting time increased 3 times when compared to clotting time of plasma incubated with two minimum coagulant dose of venom control.

**Results**

Five of the plant species included in this study showed some levels of inhibitory action against acetyl cholinesterase as indicated by their IC<sub>50</sub> values. The detailed phytoconstituent analysis including total phenol, flavanoid and flavanol are impregnated in table 2. The detailed inhibitory action of aqueous extracts of test plant species against acetyl cholinesterase was depicted in table 3. *V. negundo* and *M. indica* showed better inhibitory action against acetyl cholinesterase.

**Table 2:** Total phenolic, flavanoid and flavones content of test plant species

Plant species	Extraction and plant part	Total Phenol*	Total Flavanoid**	Total flavanol**
<i>Emblica officinalis</i>	Aqueous (Fruits)	241 ± 8.0	24 ± 4.2	21 ± 3.6
<i>Hemidesmus indicus</i>	Aqueous (Roots)	3.45 ± 0.12	2.19 ± 0.03	2.11 ± 0.04
<i>Mangifera indica</i>	Aqueous (Stem bark)	0.75 ± 0.22	6.86 ± 0.20	5.91 ± 0.19
<i>Tamarinda indica</i>	Aqueous (Seed)	20.43 ± 0.29	4.06 ± 0.06	3.84 ± 0.04
<i>Vitex negundo</i>	Aqueous (Leaves)	50.27 ± 0.19	1-.72 ± 0.16	9.61 ± 0.14

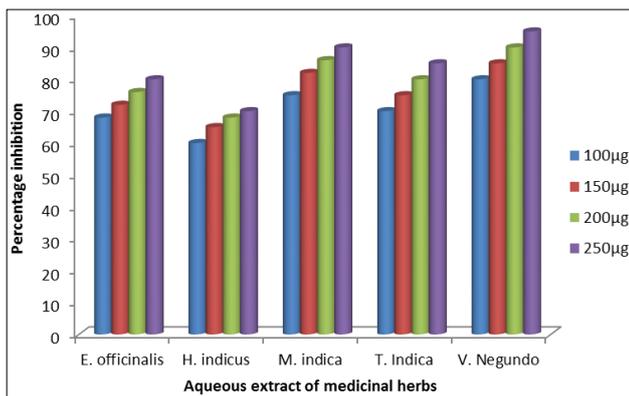
[\*Expressed as mg tannic acid/g of extract; \*\*Expressed as mg quercetin/g of extract]

**Table 3:** *In vitro* acetyl cholinesterase inhibitory action of test herbs

Plant species	Extraction and plant part	IC <sub>50</sub> (mg/l)	AChE inhibition factor (IF) IC <sub>50</sub>
Control (Galatamine)		6.62	1
<i>Emblica officinalis</i>	Aqueous (Fruits)	6.71	1.04
<i>Hemidesmus indicus</i>	Aqueous (Roots)	11.78	0.68
<i>Mangifera indica</i>	Aqueous (Stem bark)	15.29	0.54
<i>Tamarinda indica</i>	Aqueous (Seed)	12.17	0.62
<i>Vitex negundo</i>	Aqueous (Leaves)	16.43	0.57

The inhibitory action of protease activity was determined *in vitro*, and the results were presented in Figure 1. Here the extracts of *V. negundo* and *M. indica* showed better inhibitory action with the maximum inhibition of 95% and 90% respectively at the concentration of 250µg/ml.

The direct haemolytic activities of plant extract using known and test standard snake venom produced effective increase in the optical density. Among the plant extracts *V. negundo* and *M. indica* showed better results compared to other plant extracts. The Phospholipase activity was measured using indirect hemolytic assay on agarose-erythrocytes-egg yolk gel plate. Minimum indirect hemolytic dose was determined by applying increasing doses 200µg of and produced maximum of 14mm hemolytic halo. This shows that the test venom has phospholipase A2 enzyme which has the ability to cause lysis of human RBC's. The various plant extracts tested have shown good inhibition of this enzyme activity in a dose dependant manner. The reducing in haemolytic zone in the plate is reduced when concentration increased even in extracts of *V. negundo* and *M. indica* at the concentration of 200µg, there is no zone observed. The detailed patterns of reduced haemolytic zone verses plant extract concentration are depicted in table 4.



**Fig 1:** *In vitro* assay of neutralization assay (Protease activity)

**Table 4:** Inhibition of phospholipase A2 activity

Plant extract	Venom dose that gives 11mm zone	Concentration of aqueous extracts used verses haemolytic zone in mm			
		100µg	150µg	200µg	250µg
<i>Emblica officinalis</i>	200µg	11	10	10	10
<i>Hemidesmus indicus</i>		11	10	9	9
<i>Mangifera indica</i>		9	7	4	0
<i>Tamarinda indica</i>		11	10	9	8
<i>Vitex negundo</i>		8	5	2	0

## Discussion

Herbal medicines provide an alternative to anti-snake venom and are used in the folk medicine for treatment. Further reliance on medicinal plants for its safety, effectiveness, preferences, low cost and availability are to be analyzed for standardizing the medicinal plants as therapeutic agents [22]. This study highlighted the role of medicinal plants against anti-snake venom *in vitro* only; but emphasis should be taken to analyze and confirm the activity of both *in vitro* and *in vivo* according to the clinical conditions [23, 24, 25].

Screening of such herbal medicinal values for various ailments including snake bite and determination of phyto-constituents including pharmacologically efficient biomolecules may provide extensive input to the effectual treatment for snake bite [22]. The presence of flavanoids and polyphenols in our test herbal extracts showed very good effect in enzyme inhibiting and antivenom properties that is already defined in some other herbal molecules [26, 27]. Antivenom prospectiveness of five major herbal extracts was investigated by *in vitro* experimentations.

The maximum acetylcholinesterase inhibition was recorded at lowest concentration of aqueous extract of leaves of *V. negundo* and stem bark of *M. indica* (Table 2). The maximum enzyme inhibition of *Cyclea peltate* root extract against venom of *N. naja* was demonstrated [13]. Direct and indirect hemolysis properties are also well analyzed in this study (Table 3) by comparing with other studies [20, 21]. The proteolytic activity also well documented in this study (Figure 1) on comparing with other studies using different herbal extracts [13].

Few studies highlighted that on comparing with mono-herbal preparations, polyherbal preparations showed better anti-venom activity by increasing the survival period and fold of protection [12]. But requirements of higher doses may provide better inhibitory action against toxigenic enzymes [28]. The results showed that test aqueous plant extracts of fruits of *E. officinalis*, roots of *H. indicus*, stem bark of *M. indica*, seed of *T. indica* and leaves of *V. negundo* were capable of inhibiting acetylcholinesterase, protease, direct hemolytic, phospholipase and procoagulant activities.

It was concluded from the study that aqueous plant extracts of *V. negundo* and *M. indica* has antivenom activity against *N. naja* venom; further the test results were compared to commercial antivenom. This study has to be extended for the better understanding of the mechanism of anti-venom activity and neutralization. Animal models or alternative to animal experimentation like inclusion of zebra fish model are required to confirm the activity before clinical studies that claim in human beings.

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