Original Article

Anti-cobra venom activity of plant *Andrographis paniculata* and its comparison with polyvalent anti-snake venom

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Abstract

Background: To investigate the anti-cobra venom effect of alcoholic extract of *Andrographis paniculata*. **Materials and Methods:** After calculating the LD_{99} of snake venom, the venom-neutralizing ability of plant extract at the dose 1 g/kg and 2 g/kg was determined using in vitro and in vivo methods. The alleviation in the mean survival time of the animals were used to infer the antivenom property of the drug after challenging with LD_{99} of snake venom. **Results:** The ethanolic extract of plant A. paniculata significantly increases mean survival time and the protection fold, but could not protect animals from death when used alone. The higher dose, i.e., 2 g/kg was found better than that of the lower. ASV was found more effective than the plant extract. When ASV was given along with plant extract, it potentiates its effect. **Conclusion:** The observation demonstrates the anti-cobra venom activity of ethanolic extract of A. paniculata which is comparable with ASV.

Key words: *Andrographis paniculata,* anti-cobra venom, anti-snake venom,LD_{99}

INTRODUCTION

Snakebite is one of the important public health problem of tropical countries including India.[1] On an average, nearly 200 000 persons fall prey to snake bite per year in India and approximately 35 000 to 50 000 lives are lost per year.[2,3]

Indian Cobra (Naja naja) is one of the snakes commonly associated with human mortality in India. The cobra is considered very dangerous and produces systemic poisoning because rapid action of neurotoxin causes respiratory paralysis and death.[4-6]

Anti-snake venom (ASV) is a specific antidote to snake venom actions and the mainstay of treatment. Monovalent ASV is preferable to the polyvalent type since it is less hazardous to the patient and likely to be more effective in the treatment of the particular bite; however, a species diagnosis must be made before the right treatment can be chosen. Polyvalent ASV is commonly used against snakebite, but it is expensive and composed of antibodies from immunized animals; hence, there are chances of adverse reactions due to activation of immune system in about 20% of patients.[7-10]

In contrast to the difficulty of availability of modern treatment in large areas of developing world where venomous snakes occur, numerous plant species are used as the folk medicine to treat snake bites. Many Indian medicinal plants are recommended for the treatment of snake bite activity. *Andrographis paniculata*, is one of the plants that has long been used in traditional herbal medicine against snakebite.[11-14] However, only few attempts have been made to correlate scientifically and also, comparison of its anti-cobra venom activity with ASV.

So, an attempt was made to screen the anti-cobra venom potential of the plant *A. paniculata* and further to compare its anti-cobra venom activity with polyvalent ASV.

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MATERIALS AND METHODS

Collection of the plant materials

The plant materials were brought from Tamil Nadu and then authenticated by the local botanist of Science College. The plant materials were then cultivated during rainy phase of summer season (June and July) in the land near hospital. The plants at flowering stage, i.e., after 90 to 120 days after sowing, were cut at the base leaving 10 to 15 cm stem for plant regeneration.

Preparation of extract

Fresh plants were collected, carefully cleaned, dried in shade, powdered, and stored in airtight containers until it was used for further studies. Alcoholic extract was prepared according to the procedure reported by Mahanta and Mukharjee.[15] Forty grams of dried powder of each plant was macerated in 95% of ethanol overnight. Then, it was packed in the timble of Soxhlet apparatus and was extracted using 95% ethanol refluxing at 60 to 80°C, which yielded an extract which was dark brown in color. The stock extract was preserved in airtight glass container and kept inside the refrigerator at 4°C.

Venom sample

Lyophilized venom sample of *Naja naja* was purchased from Haffkine Institute, Parel, Mumbai, and were preserved at 2 to 8°C for future use (Letter No. HI/2NS/5617/26).

Anti-snake venom

The polyvalent ASV was purchased from the local medical store. It was manufactured by Biological E limited having M.L no. 02/HD/AP/98/V/R, MFD Sep 2007 and EXP Aug 2010. Each ml of ASV neutralizes 0.6 mg of standard cobra venom (*Naja naja*), 0.45 mg of standard Krait venom (*Bungarus caeruleus*), 0.6 mg of standard Russell's viper venom (*Vipera russelli*), and 0.45 mg of standard Saw-scaled viper venom (*Echis carinatus*).

Test animals

Male Swiss albino mice weighing 20 to 25 g were used for the studies. All the animals were conditioned in standard cages. They were kept in a 12/12 hour light dark cycle and fed on standard laboratory chow and water *ad libitum*.

Ethical clearance

Ethical clearance was taken from Institutional Animal Ethics Committee of the institute where the research was conducted (MGIMS/IAEC/4/2008).

Calculation of LD_{99} of Indian Cobra *(Naja naja)* venom

Lethal dose 99 is defined as the least amount of venom (dry weight in grams) injected intraperitoneally (i.p.) to animals resulting in the 99% death of animals within 24 hours. The method reported by Turner was adopted for determination of LD_{99}.[16]

The *Naja naja* snake venom was dissolved in distilled water (DW) and given to mice i.p. in graded doses and mortality was recorded for 24 hours. The lowest dose taken was 0.28 mg/kg. Five animals were taken in each group.

Calculation of ED_{50} of anti-snake venom against Indian Cobra venom

Effective dose 50 is defined as least amount of ASV injected i.p. to animals resulting in the 50% survival of animals within 24 hours.

The LD_{99} of *Naja naja* snake venom was given to mice i.p., then ASV was given immediately after that in graded doses and mortality was recorded for 24 hours. Five animals were taken in each group.

Acute toxicity of Indian Cobra venom and its neutralization by plant extract and anti-snake venom

Animals were divided into six groups of six animals. Each animal in all the groups were administered LD_{99} of snake venom i.p. Group 1 received DW and was considered as control. Group 2 and group 3 received plant extract at the dose of 1 g/kg and 2 g/kg i.p. Group 4 received ED_{50} of ASV i.p. and served as the standard control. Group 5 received plant extract at the dose of 1 g/kg plus ED_{50} ASV, while Group 6 had received plant extract at the dose of 2 g/kg plus ED_{50} ASV. The plant extract was dissolved in DW. ASV and plant extract were given 5 minutes after the dose of snake venom. In all the groups, the duration of survival and animal survived were recorded for 24 hours. All the groups received same volume of preparations.

Neutralization of the lethal venom effect of Indian Cobra by Alam and Gome's method

Neutralization test described by Alum and Gomes was followed.[17] Animals were divided into four groups of six animals each. LD_{99} of snake venom was mixed with plant extracts at the dose of 1 g/kg and 2 g/kg, then the mixture was incubated for 1 hour at 37°C and centrifuged at 2 000 rpm for 10 minutes. The supernatant was injected i.p. into mice. The duration of survival and animal survived were recorded for 24 hours after admixture injection of venom. Group 2 received 1 g/kg plant extract. Group 3 received 2 g/kg of plant extract, while Group 1 received DW i.p. and served as control. Group 4 received admixture of snake venom and ASV and served as standard. All the groups received same volume of preparations.

Neutralization of the lethal venom effect of *Naja naja* by Martz's method

Neutralization test as described by Martz was followed with some modifications.[18] Animals were divided into three groups of six animals each. The plant extract dissolved in DW was given orally for 5 days. On day 5, after 30 minutes of last dose, the LD_{99} of snake was administered. The duration of survival and animal survived were recorded for 24 hours after injection of venom. Group 2 received 1 g/kg of plant extract. Group 3 received 2 g/kg of plant extract, while Group 1 received DW orally and served as control. All the groups received same volume of preparations.

Blinding

All the experiments were singly blinded in which one of the postgraduate student recorded survival time and animals survived in each experiments.

STATISTICAL ANALYSIS

The statistical analysis was done using one way analysis of variance (ANOVA) using unpaired student's *t* test. *P* value <0.05 was considered statistically significant.

RESULTS

Calculation LD_{99} of *Naja naja* venom

Lethality data of *Naja naja* venom is shown in Table 1. LD_{99} was calculated by probit analysis. The LD_{99} of *Naja naja* venom from this study was 3.162 mg/kg. LD_{50} (median lethal dose) was also calculated from the same data and was found to be 0.7943 mg/kg [Table 1 and Figure 1].

ED_{50} of anti-snake venom against *Naja naja* venom

ED_{50} was calculated by probit analysis. The ED_{50} of ASV against *Naja naja* venom from this study was 0.0086 ml/g [Table 2 and Figure 2].

Acute toxicity of *Naja naja* venom and its neutralization by plant extract and antivenom

The *Naja naja* venom at the dose 3.162 mg/kg (LD_{99}) produces 100% death in mice. The ethanolic extract of plant *A.paniculata* significantly increases mean survival time and the protection fold but could not protect animals from death when used alone.

The plant extract when used alone at the dose of 2 g/kg was found more effective against *Naja naja* venom showing mean survival 4.575 hours, as compared with 1.817 hours shown by plant extract at the dose of 1 g/kg.

The ASV was found more effective as compared with the plant extract showing mean survival of 19.975 hours and complete survival of one mouse. Increased mean survival time and protection fold was found when ASV was administered along with plant extract.

The plant extract at the dose 2 g/kg was found more effective when administered along with snake venom showing mean survival of 22.783 hours and also complete survival of two mice as compared with one mouse when 1 g/kg was administered [Table 3].

Neutralization of the lethal venom effect of snake *Naja naja* by Alam and Gome's method

The LD_{99} of *Naja naja* venom which was mixed with control produces 100% death of mice. When Snake venom was mixed with ethanolic extract of plant *A. paniculata* , mean survival time and the protection fold were significantly increased but could not protect animals from death.

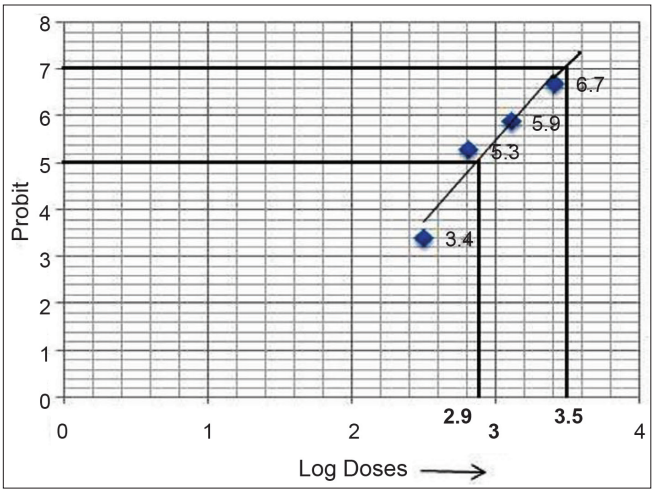

Figure 1: Calculation LD_{99} of Naja naja venom in mice receiving various doses of venom by Turner's method (n = 5)

Table 1: % death of mice receiving various doses of *Naja naja* venom (n = 5)

Dose mg/kg	Dose µgm/kg	Log dose	Dead/total	Dead %	Corrected %*	Probit
0.28	280	2.4472 ≈ 2.5	0/5	0	5	3.36 ≈ 3.4
0.56	560	2.7482 ≈ 2.8	3/5	60	60	5.25 ≈ 5.3
1.12	1112	3.0492 ≈ 3.1	4/5	80	80	5.84 ≈ 5.9
2.24	2240	3.3502 ≈ 3.4	5/5	100	95	6.64 ≈ 6.7

Corrected formula*: For the 0% dead: 100 (0.25/n) = 100 (0.25/5) = 5, For the 100% dead: 100 [(n-0.25)/n] = 100 [(5-0.25)/5] = 95, n is the number of animals in the group

Table 2: % death of mice receiving various doses of ASV after giving LD_{99} of *Naja naja* venom (n = 5)

Average wt (g) *	Average dose of ASV (ml) †	Log dose	Survived/total	Survived %	Corrected% ‡	Probit
24.2	0.081	1.9085 ≈ 1.9	0/5	0	5	3.36 ≈ 3.4
22.8	0.150	2.1761 ≈ 2.2	2/5	40	40	4.75 ≈ 4.8
21.8	0.285	2.4548 ≈ 2.5	4/5	80	80	5.84 ≈ 5.9
23.2	0.610	2.7853 ≈ 2.8	5/5	100	95	6.64 ≈ 6.7

Average wt*: Addition of weight of all five mice in one group who received ASV at same dose/5, Average dose†: Addition of ASV received after receiving snake venom by all five mice in one group/5, Corrected formula‡: For the 0% survived: 100 (0.25/n) = 100 (0.25/5) = 5, For the 100% survived: 100 [(n-0.25)/n] = 100 [(5-0.25)/5] = 95, n is the number of animals in the group

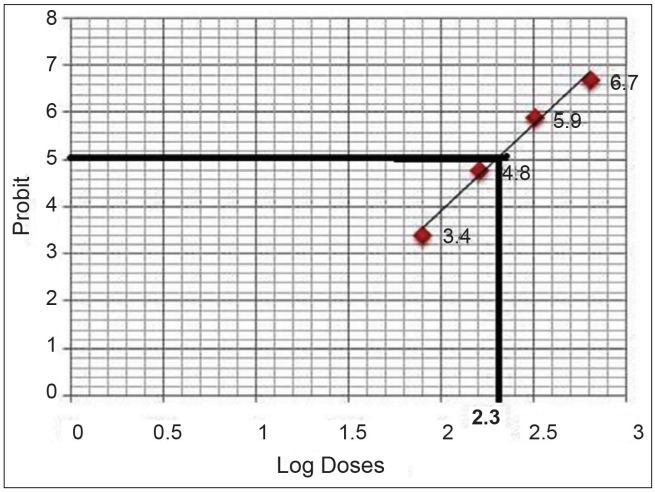

Figure 2: Calculation ED50 of ASV in mice receiving various doses of venom (n = 5)

The plant extract when used at the dose of 2 g/kg was found more effective against *Naja naja* venom showing mean survival 8.175 hours, as compared with 3.475 hours shown by plant extract at the dose of 1 g/kg.

The antivenom was found more effective as compared with the plant extract showing mean survival of 21.733 hours with complete survival of two mice [Table 4].

Neutralization of the lethal venom effect of *Naja naja* by Martz's method

Group 1 received DW for 5 days before administration of *Naja naja* venom at the dose 3.162 g/kg. It produced 100% death of mice. Group 2 that received ethanolic extract of plant *A. paniculata* significantly increases mean survival time and the protection fold but could not protect animals from death. The plant extract in the dose of 2 g/kg was found to be more effective against *Naja naja* venom showing mean survival 6.008 hours, as compared with 3.175 hours shown by plant extract at the dose of 1 g/kg [Table 5].

DISCUSSION

The toxins of Indian Cobra venom are composed of neurotoxin, cardiotoxin, enzymes, and proteins. The victim may die from respiratory paralysis which is the major cause of death. ASV and assisted ventilation can save life in many cases.[4,19]

However, polyvalent antivenom carries a risk of severe adverse reactions, and other problems such as difficulty to manage and usage, variety of dosage, and high cost. Furthermore, antivenom sometimes does not provide enough protection against snake envenomation, especially local poisoning.[20,21]

The use of plants against the effect of snakebite has long been recognized, even in modern times. Only for last 20 years, it has merited to closer scientific attention. Although quite a number of reports from different geographic areas mention plants reputed to neutralize the action of snake venom, only a few attribute such activity to certain chemical compounds identified in them, and even less are concerned with a possible mechanism of action.

In India, many plants are recognized against snake envenomation. *A. paniculata* was used by many folk healers, especially in the southern region of India. Also, there are many Ayurvedic preparations for snake bites in which *A. paniculata* is one of the constituent.[22-27]

In this study, we used lyophilized (freeze dried) snake venom because venom is easily perishable and in the solid form, it is easy to handle. LD_{99} of *Naja naja* was calculated because the lethality of the same snake venom varies from place to place.

It was observed that the ethanolic extract of plant *A. paniculata* when given to the mice after they received snake venom of *Naja naja* significantly increased mean survival time and protection fold but could not protect mice from death when used alone and the results were found better when it was used at higher dose. Also, when it is used along with ASV, it increased potency of the ASV. This could be possible due to inactivation or precipitation of active venom components by the plant extract. This result is similar to previous studies. Fatepur and Gawade and

Table 3: Mean survival time, protection fold, survived animals, and % survival against the LD_{99} of *Naja naja* venom when challenged by plant extract and ASV immediately after venom administration

Groups	Mean survival time (hr)	Protection fold	Total animal survival/total no. of animals in group	% survival
Group 1 LD_{99}SV+ DW	0.492 ± 0.074	-	0/6	0
Group 2 LD_{99}SV+ PE 1	1.817 ± 0.352*	3.70	0/6	0
Group 3 LD_{99}SV+ PE 2	4.575 ± 0.423*	9.30	0/6	0
Group 4 LD_{99}SV+ ASV	19.975 ± 2.609*	40.60	1/6	16.67
Group 5 LD_{99}SV+ASV+PE 1	20.967 ± 2.097*	42.61	1/6	16.67
Group 6 LD_{99}SV+ DW+PE 2	22.783 ± 0.951*	46.31	2/6	33.33

Results were expressed in Mean ± SD; unpaired student "t" test; *P<0.001;, LD_{99}SV: LD_{99} of *Naja naja* snake venom, PE1: Plant extract of *Andrographis paniculata* at the dose of 1 g/kg, PE2: Plant extract of *Andrographis paniculata* at the dose of 2 g/kg, DW: Distilled water; ASV: The ED_{50} of antivenom

Table 4: Mean survival time, protection fold, survived animals, and % survival against the LD_{99} of *Naja naja* venom when mixed with plant extract and ASV

Groups	Mean survival time (hr)	Protection fold	Total animal survival/total no. of animals in group	% survival
Group 1 LD_{99}SV+ DW	0.667 ± 0.320	-	0/6	0
Group 2 LD_{99}SV+ PE 1	3.475 ± 0.654*	5.21	0/6	0
Group 3 LD_{99}SV+ PE 2	8.175 ± 0.667*	12.26	0/6	0
Group 4 LD_{99}SV+ ASV	21.733 ± 2.017*	32.58	2/6	33.33

Results were expressed in Mean ± SD; unpaired student "t" test; *P<0.001; LD_{99}SV: LD_{99} of *Naja naja* snake venom. PE1: Plant extract of *Andrographis paniculata* at the dose of 1 g/kg, PE2: Plant extract of *Andrographis paniculata* at the dose of 2 g/kg, DW: Distilled water; ASV: The ED_{50} of antivenom.

Table 5: Mean survival time, protection fold, survived animals, and % survival against the LD_{99} of *Naja naja* venom when challenged by plant extract orally for 5 days

Groups	Mean survival time (hr)	Protection fold	Total animal survival/total no. of animals in group	% survival
Group 1 LD_{99}SV+ DW	0.592 ± 0.231	-	0/6	0
Group 2 LD_{99}SV+ PE 1	3.175 ± 0.398*	5.36	0/6	0
Group 3 LD_{99}SV+ PE 2	6.008 ± 0.404*	10.15	0/6	0

Results were expressed in Mean ± SD; unpaired student "t" test; *P<0.001; LD_{99}SV: LD_{99} of *Naja naja* snake venom, PE1: Plant extract of *Andrographis paniculata* at the dose of 1 g/kg, PE2: Plant extract of *Andrographis paniculata* at the dose of 2 g/kg, DW: Distilled water; ASV: The ED_{50} of antivenom.

Nazimuddin *et al.* reported that ethanolic extract could inhibit lethal activity of *Naja naja* venom.[28,29]

The pharmacological properties of snake venom are mainly associated with proteins, particularly enzymes. Venom of *Naja naja* is also the mixture of different proteins. It contains powerful postsynaptic neurotoxins which are low molecular weight and diffuses rapidly through blood stream. It also contains toxic phospholipase A_2 with presynaptic neuromuscular blocking activity. PLA_2 is almost invariably the most toxic component of the venom and responsible for wide range of pharmacological effects, including neurotoxicity, cardiotoxicity, heamotoxic, and damage to biological membranes.[30,31]

Naja naja venom kills the animal by producing respiratory paralysis attributable to their content of α neurotoxins

binding to α subunit of the acetylcholine receptor at the neuromuscular junction.

It was observed that extract of the plant *A. paniculata* provides some protection against the lethal dose of venom. Certain naturally occurring substances such as sitosterol, pentacyclic terpines, nitro compounds (aristolochic acid), cinnamic acid derivatives, curcuminoids, polyphenolic compounds, and flavonoids are known compounds possessing protein-binding and enzyme-inhibiting properties. The leaves of *A. paniculata* contains andrographolide, the active constituent of which is diterpene and is responsible for ASV property by modifying the actions of proteins, and enzymes also inhibit snake venom phospholipase A_2 activities.

In the other experiment in which snake venom of *Naja naja* and extract of the plant were used for study when mixed outside also significantly increased the mean survival time and protection fold but could not protect mice from death. When used alone, the results were found better when it was used at higher dose. This also suggests that the plant extract neutralizes the snake venom.

ASV was found better than plant extract in increasing the mean survival time and protection fold and also protected two animals from death.

When the plant extract was given to the mice orally for 5 days, it also showed the protection from the snake venom. This suggests that it has some prophylactic value. But the exact cause could not be ascertained. These results were also similar to the study done by Fatepur and Gawade on the preliminary screening of herbal plant extract for ASV activity against common sea snake.

SUMMARY AND CONCLUSION

Thus, it can be concluded from the study that *A. paniculata* plant extract has antivenom activity against *Naja naja* venom. Results are comparable with the antivenom. Further elaborative work is necessary for the better understanding of the mechanism of venom inhibition. Detailed clinical studies in this direction are required to potentiate this claim in human beings.

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