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Studies on antivenom activity of *Andrographis paniculata* and *Aristolochia indica* plant extracts against *Daboia russelli* venom by *in vivo* and *in vitro* methods

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Abstract: Methanolic extracts of *Andrographis paniculata* and *Aristolochia indica* plants were tested for antivenom activity against *Daboia russelli* venom. Both plant extracts effectively neutralized the *D. russelli* venom induced lethal activity. About 0.15 mg of *A. paniculata* and 0.14 mg of *A. indica* plant extracts were able to completely neutralize the lethal activity of $2LD_{50}$ of *D. russelli* venom. Various pharmacological activities including edema, haemorrhagic, coagulant, fibrinolytic and phospholipase activities were studied and these pharmacological activities were significantly neutralized by both the plant extracts. The above observations confirmed that *A. paniculata* and *A. indica* plant extracts possess potent snake venom neutralizing capacity and could potentially be used for therapeutic purposes in case of snakebite envenomation.

Keywords: AntiVenom, plant extracts, snake bite.

Introduction

Snakebite is a major health hazard that leads to high mortality rate especially in India. The common poisonous snakes found in India are Cobra (*Naja naja*), Krait (*Bangarus Caeruleus*), Russell's viper (*Daboia russelli*) and Saw Scaled Viper (*Echis Carinatus*) (Bawaskar, 2004). About 35,000 to 50,000 people die of snakebite every year in India. *Vipera russellii* and *Naja kaouthia* are the common snakes found throughout India and a large number of deaths occur due to envenomation by these snakes (Alam & Gomes, 2003). Antivenom immunotherapy is the only specific treatment against snake venom envenomation. There are various side effects of antivenom such as anaphylactic shock, pyrogen reaction and serum sickness. Most of these symptoms may be due to the action of high concentrations of nonimmunoglobulin proteins present in commercially available hyper immune antivenom (Maya Devi *et al.,* 2002). Over the years many attempts have been made for the development of snake venom antagonists from plants sources. Several medicinal plants, which appear in old drug recipes or which have been passed on by oral tradition, are believed to be snakebite antidotes and are recommended for the treatment of snakebite (Alam & Gomes, 2003). Some of these plant extracts has shown antivenom property on land snakes and their possible mechanism of action have been suggested. In almost any part of the world, where venomous snakes occur, numerous plant species are used as folk medicine to treat snakebite. The present investigation explored the *D. russelli* venom neutralizing activity of *Andrographis paniculata* and *Aristolochia indica* plant extracts by *in vivo* and *in vitro* methods.

Materials and methods

Venom and experimental animals

The freeze-dried snake venom powder of *Daboia russelli* was obtained from Irula's Snake Catchers Industrial Co-operative Society Limited, Chennai and was stored at 4°C. Male inbreed Swiss albino mice 18-20 g were used for efficacy studies. Institutional Animal Ethics Committee clearance at Institute of Vector Control and Zoonooses, Hosur, was obtained to conduct the experiment.

Medicinal plants and preparation of extracts

Andrographis paniculata and *Aristolochia indica* plants were obtained from RVS Ayurveda College, Sulur. The whole plant material was dried in shade and the air-dried plant was ground and extracted first by refluxing with petroleum ether (50-60°C, for 72 h) and then with methanol (60-80°C for 72 h). The methanol extract was concentrated in vaccuo and kept in a desiccator at room temperature for further use. Before use, it was dissolved in saline and centrifuged at 2000 rpm for 10 min at room temperature. The supernatant was used for further investigation and kept at 4°C. The plant extracts were expressed in terms of dry weight.

In vivo assessment of venom toxicity and anti-venom effect of plant extracts

Lethal toxicity: The median lethal dose (LD_{50}) of *D. russelli* venom was determined according to the method developed by Theakston and Reid 1983. Various doses of venom in 0.2 ml of physiological saline was injected into the tail vein of mice, using groups of 3-5 mice for each venom dose. The LD_{50} was calculated with the confidence limit at 50% probability by the analysis of deaths occurring within 24 h of venom injection. The anti-lethal potentials of *A. paniculata* and *A. indica* plant extracts were determined against $2LD_{50}$ of *D. russelli* venom. Various amount of plant extracts (µl) were mixed with $2LD_{50}$ of venom sample and incubated at 37°C for 30 minutes and then injected intravenously into mice. 3-5 mice were used at each antivenom dose. Control mice received same amount of venom without antivenom (plant extracts). The median Effective Dose (ED_{50}) calculated from the number of deaths within 24h of injection of the venom/antivenom mixture. ED_{50} was expressed as µl antivenom/mouse and calculated by probit analysis.

Edema- forming Activity: The minimum edema-forming dose (MED) of *D. russelli* venom was determined by the method of Lomonte *et al.* (1993) and Camey *et al.* (2002). MED was defined as the least amount of venom which when injected subcutaneously into mice footpad results in 30% edema with in 6 hours of venom injection. The thickness of each footpad was measured every 30 min after venom injection with a low-pressure spring caliper (Rojas *et al.*, 2005). The ability of both plant extracts in

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neutralizing the edema-forming activity were carried out by pre-incubating the constant amount of venom and various dilutions *A. paniculata* and *A. indica* plant extracts for 30 minutes at 37°C. Mice were injected subcutaneously in the right footpad with 50µl of the mixtures, containing venom/plant extracts, whereas the left footpad received 50µl of PBS alone. Control mice were injected with venom in the right footpad and 50µl of PBS in the left footpad. 1 hour after injection edema was evaluated as described by Yamakawa *et al.* (1976). Edema was expressed as the percentage increase in thickness of the right footpad compared to the right footpad of the control mice.

Haemorrhagic activity: The minimum haemorrhagic dose (MHD) of *Daboia russelli* venom was determined by the method described by Theakston and Reid, 1983. The minimum haemorrhagic dose was defined as the least amount of venom which when injected intradermaly (i.d.) into mice results in a haemorrhagic lesion of 10mm diameter in 24 hours. Neutralization of the haemorrhagic activity was estimated by mixing a fixed amount of venom with different amounts plant extracts. The plant extract-venom mixture was incubated at 37°C for 1 h and 0.1 ml of the mixture was injected intradermaly into mice. The haemorrhagic lesion was estimated after 24 h.

Defibrinogenating activity: The minimum defibrinogenating dose (MDD) of *Daboia russelli* venom is defined as the minimum amount of venom which when injected i.v. into mice causes incoagulable blood 1 h later (Theakston & Reid, 1983). Neutralization of this activity was estimated by mixing different amount of venom with fixed amounts of plant extract, incubating at 37°C for 1 h, and centrifuging. The supernatant was injected i.v. into mice and the nature of the blood observed after 1 h.

In vitro assessment of venom toxicity and anti-venom effect of plant extracts

Phospholipase activity: Phospholipase A2 activity was measured using an indirect hemolytic assay on agarose-erythrocyte-egg yolk gel plate by the methods described by Gutierrez *et al*., 1988. Increasing doses of *Daboia russelli* venom (µg) were added to 3mm 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 $CaCl_2$. Slides were incubated at 37˚C overnight and the diameters of the hemolytic halos were measured. Control wells contained 15µl of saline. The minimum indirect hemolytic dose (MIHD) corresponds to a dosage of venom, which produced a hemolytic halo of 11mm diameter. The efficacy of antivenom (plant extracts) in neutralizing the phospholipase activity was determined by mixing constant amount of venom (µg) with various amount of plant extracts (µl) and incubated for 30 minutes at 37°C. Then, aliquots of 10µl of mixtures were added to wells in agarose-egg yolk-sheep erythrocyte gels. Control samples contain venom without plant extracts. Plates were incubated at 37°C for 20 h. Neutralization was expressed as the ratio of mg antibodies/mg venom which could reduce the diameter of the hemolytic halo by 50% when compared to the effect induced by venom alone.

Procoagulant activity: Procoagulant activity was assayed according to the method described by Theakston and Reid, 1983 as modified by Laing *et al*., 1992. Various amounts of venom dissolved in 100µl PBS (pH 7.2) was added to human citrated plasma at 37°C. Coagulation time was recorded and the minimum coagulant dose (MCD) was determined as the venom dose, which induced clotting of plasma within 60 seconds. Plasma incubated with PBS alone served as control. In neutralization assays constant amount of venom was mixed with various dilutions of plant extracts. The mixtures were incubated for 30 minutes at 37°C. Then 0.1ml of mixture was added to 0.3ml of citrated plasma and the clotting times recorded. In control tubes plasma was incubated with either venom alone or plant extracts alone. Neutralization was expressed as effective dose (ED), defined as the ratio µl antivenom (plant extracts)/mg venom at which the clotting time increased three times when compared with clotting time of plasma incubated with two MCD of venom alone.

Fibrinolytic activity: A modified plaque assay was used (Rojas *et al*., 1987). The minimum fibrinolytic concentration was defined as the concentration of venom that induced a fibrinolytic halo of 10mm diameter. Neutralization experiments were performed by incubating a constant amount of venom with various amounts of plant extracts at 37°C for 1 h. After incubation, the mixture was applied to the wells in the plaque. After 18 h of incubation at 37°C, fibrinolytic halos were measured.

Statistical Analysis

Statistical evaluation was performed using XL Stat 2007 and SPSS 10 software. $P < 0.005$ was considered statistically significant.

Results

The antivenom potential of *A. paniculata* and *A. indica* plant extracts were tested against *Daboia russelli* venom using in vivo and in vitro methods. The lethal toxicity (LD_{50}) of *D. russelli* venom was assessed using Balb/c strain mice. About 8 µg of *D. russelli* venom was found to be LD_{50} for 18g of mice. The neutralization of lethality was done by mixing constant amount of venom ($2LD_{50}$) with various dilutions of *A. paniculata* and *A. indica* plant extracts and incubated at 37°C for 30 minutes prior to injection. We found that 0.15 mg of *A. paniculata* and 0.14 mg of *A. indica* plant extracts were able to completely neutralize the lethal activity of $2LD_{50}$ of *D. russelli* venom (Table 1, Fig. 1).

Table 1. Neutralization of D. russelli venom induced lethality by plant extracts

Plant extracts	Dose of *D. russelli* venom (µg)	Neutralization of venom by plant extracts (ED_{50} in mg)
A. paniculata	16 ($2LD_{50}$)	0.15 mg
A. indica	16 ($2LD_{50}$)	0.14 mg

In edema forming activity, mice immunized with *Daboia*

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russelli venoms showed an increase in footpad thickness. About 4µg of Russell's viper venom induced edema formation within 3h which is considered as 100% activity. The edema was reduced up to 30% when 2.7 mg of plant extracts per mg venom was given. No further reduction in the percentage of edema was observed even when there was an increase in antivenom dose (Fig. 2).

In the case of hemorrhagic activity, Russell's viper venom did not produced any visible hemorrhagic spot. In phospholipase activity (PLA_2), *D. russelli* venom able to produce hemolytic haloes in agarose-sheep erythrocytes gels. About 10µg of Russell's viper venom produced 11mm diameter hemolytic halo, which is considered to be 1U (U/10µg). This shows that *D. russelli* venoms have the enzymes (PLA_2) that has the ability to lyse sheep RBC's. Both plant extracts were capable of inhibiting PLA_2 dependent hemolysis of sheep RBC's induced by *D. russelli* venom in a dose dependent manner. We found that that 0.13mg of *A. paniculata* and 0.11mg of *A. indica* plant extracts were able to completely inhibit PLA_2 dependent hemolysis of sheep RBC's induced by *D. russelli* venom (Table 2). The minimum coagulant dose (MCD) was determined as the venom dose inducing clotting of plasma in 60s. About 120µg of Russell's viper venom clotted human citrated plasma within 60s. In the neutralization assay, the absence of clot formation shows the neutralizing ability of both plant extracts. We found that 1.8 mg of *A. paniculata* and 1.6 mg of *A. indica* plant extracts were able to completely neutralize coagulant activity. High dose of venom caused rapid clotting that required very high dose of antivenom to neutralize. *A. paniculata* and *A. indica* plant extracts were effectively antagonised the Russell's viper venom-induced defibrinogenating activity. The effective dose were found to be 1.3 mg for *A. paniculata and* 1.1 mg for *A. indica* plant extracts. The viper venom-induced fibrinolytic effect was effectively antagonized by the plant extracts (*A. paniculata* and *A. indica*). The ED50 of *A. paniculata* and *A. indica* were found to be 0.6 and 0.5 mg, respectively. Both plant extracts were capable of inhibiting fibrinolytic activity induced by *D. russelli* venom. We found that that 0.14mg of *A. paniculata* and 0.11mg of *A. indica* plant extracts were able to completely inhibit fibrinolytic activity (modified plaque assay) induced by *D. russelli* venom.

Discussion

Snakebite is a major health hazard that leads to high mortality rate especially in India. *Vipera russelli* and *Naja kaouthia* are the common snakes found throughout India and a large number of deaths occur due to envenomation by these snakes. Antisnake venom remains the specific antidote for snake venom poisoning. This antisnake venom is usually derived from horse sera. They contain horse immunoglobulins, which frequently caused complement mediated side effects, and other proteins that cause serum sickness and occasionally, anaphylactic shock. Although, use of plants against the effects of snakes bite has been long recognized, more scientific attention has been given since last 20 years (Santosh *et al*., 2004). Many Indian medicinal plants are recommended for the treatment of snakebites (Alam *et al*., 2003). In this study we examined the antivenom potential of *A. paniculata* and *A. indica* plant extracts

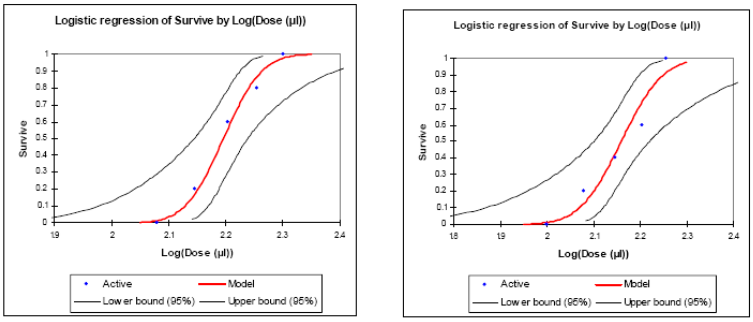

Fig 1 - Dose response curve for Neutralization of Lethality by a) Andrographis paniculata and b) Aristolochia indica Plant extracts against *Daboia russelli* venom in experiments involving preincubation of venom (2 x LD_{50}) and various concentrations of antivenoms (Plant Extracts). The median effective dose for *Daboia russelli* venom was 157.0µl (139.45 – 173.16) for *Andrographis paniculata and* 144.26µl (126.06-165.69) for *Aristolochia indica Plant extracts* .

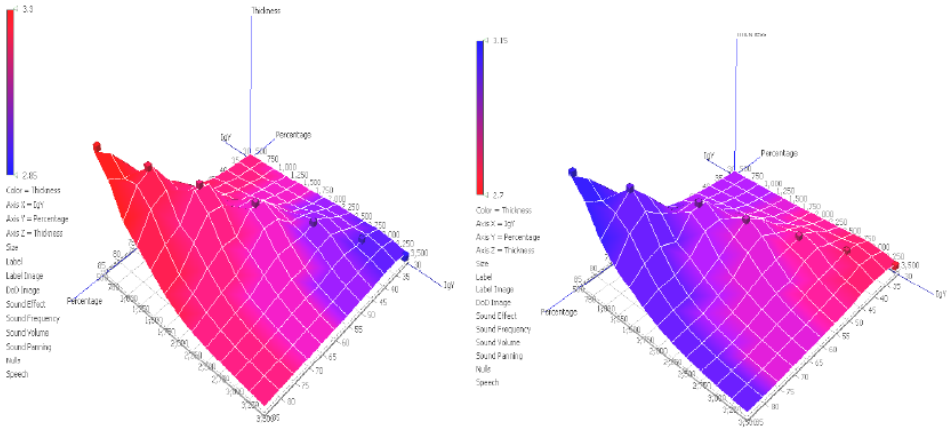

Fig 2 - Neutralization of Edema induced by Russell's viper venom by a) Andrographis paniculata and b) Aristolochia indica Plant extracts in experiments with pre-incubation. Various mixtures of venom and antivenoms were incubated and tested in the foot pad assay. Edema was assessed 1 hour after injection and expressed as percentage. Edema induced in control mice (venom alone) was considered as 100% activity. Results presented as mean ± SE (N=3). P<0.005 at all antivenoms/venom ratios

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against *D. russelli* venom. The neutralization ability of snake antivenoms is still assessed by the traditional *in vivo* lethality assay (minimum effective dose ED_{50}), comparable to those used for bacterial antitoxins, usually performed in mice (WHO, 1981). Thus various pharmacological activities like lethality, edema forming activity, hemorrhagic activity, fibrinolytic activity, PLA_2, procoagulant activity caused by *D. russelli* venom were carried out. Neutralization of these pharmacological effects was carried out using *A. paniculata* and *A. indica* plant extracts. Neutralization studies can be performed by incubating of venom and plant extracts prior to testing (pre-incubation method). The results showed that the both plant extracts were capable of neutralizing the lethality induced by the venom. The *D. russelli* venom showed the presence of PLA_2 enzymes by means of producing hemolytic haloes in indirect hemolytic assays. Both plant extracts were capable of inhibiting PLA_2 dependent hemolysis of sheep RBCs in a dose dependent manner. Edema-forming activity was assessed for *D. russelli* venom and both plant extracts were found to be effective in neutralization of edema induced by venoms. There was a significant decrease in the edema (footpad thickness) when there was an increase in the antivenom (plant extract) dose. Procoagulant activity induced by *D. russelli* venom was studied using human citrated plasma and *A. paniculata* and *A. indica* plant extracts were found to be effective in the neutralization of procoagulant activity. *An. paniculata* and *A. indica* plant extracts effectively antagonised the Russell's viper venom-induced defibrinogenating activity and fibrinolytic activity. In conclusion the present experimental results indicate that *A. paniculata* and *A. indica* plant extracts were effective in neutralizing the toxic effects of the *D. russelli* venom.

Table 2. Phospholipase activity of D.russelli venom and its neutralization by plant extracts

Plant extracts	Dose of *D. russelli* venom (µg)	Neutralization of venom by plant extracts (ED_{50} in mg)
A. paniculata	10 (1 Unit)	0.13 mg
A. indica	10 (1 Unit)	0.11 mg

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