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RESEARCH ARTICLE

In Vivo and *In Vitro* Studies on Neutralizing Effects of Acorus *calamus* and Withania somnifera root extracts against *Echis carinatus* venom

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ABSTRACT

Neutralization effects of *Acorus calamus* and *Withania somnifera* root extracts were tested against *Echis carinatus* venom. Both plant extracts were effectively neutralized the various pharmacological activities induced by *Echis carinatus* venom. About 0.14 mg of *Acorus calamus* and 0.16 mg of *Withania somnifera* root extracts were able to completely neutralize the lethal activity of $2LD_{50}$ of *Echis carinatus* venom. Various pharmacological activities like haemorrhagic, coagulant, edema and phospholipase activities were effectively neutralized by both plant extracts. The above observations confirmed that *both* plant extracts possess potent snake venom neutralizing compounds, which inhibit the activity of *Echis carinatus* venoms.

Keywords: *Echis carinatus Venoms, Acorus calamus*, *Withania somnifera***,** *Plant extracts, lethality, PLA2*

Snake bite is a serious medico-legal problem particularly among the forest workers and agriculturists in rural India. There are over 2000 species of snakes in the world and about 216 species exists in India, of which 52 are Venomous [1]. 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*) [2]. In India, about 15,000 persons are affected every year by snake envenomation. *Echis carinatus* (Saw-scaled viper) is responsible for a large number of snake bite case, reaching 95% of envenomations in the state of Jammu [3]. Antivenom immunotherapy is the only specific treatment against snake venom envenomation.

Antiserum development in animal is time-consuming, expensive and requires ideal storage condition. Over the years, many attempts have been made for the development of snake venom antagonists especially from plants sources. Extracts from plants have been used among traditional healers, especially in tropical areas where there are plentiful sources for snakebite therapy for a long time [4]. In modern science, there have been many attempts to study these plants to clarify their effectiveness [5, 6]. India has a rich tradition of the usage of medicinal plants. Many Indian medicinal plants are recommended for the treatment of snakebite [7]. Methanolic extracts of Andrographis paniculata and Aristolochia indica plant extracts possess potent snake venom neutralizing capacity and could potentially be used for therapeutic purposes in case of snakebite envenomation [8]. Aqueous extract of *Mimusa pudica* root possesses compounds, which inhibit the activity of *Naja naja* and *Bangarus caerulus* venoms. He also reported that aqueous extracts of *M. pudica* root possess compounds, which inhibit the activity of *Russell's viper and Saw scaled viper* venoms [9, 10]. Aqueous extracts of *Mucuna pruriens* seeds possess compounds, which inhibit the activity of cobra and krait venoms. [11]. The present investigation explored the *Neutralization effects of Acorus calamus* and *Withania somnifera* root extracts against *Echis carinatus* venom using *in vivo* and *in vitro* methods.

MATERIALS AND METHODS

Venom and Experimental animals

The free-dried snake venom powder of *Echis carinatus* 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 gm) were used for the studies of venom toxicity and in the experiments of venom neutralization. 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

Acorus calamus and *Withania somnifera* plants were obtained from Nehru Herbal Gardens, Coimbatore and the extracts were prepared by the method of Uhegbu et al. [12] using distilled water as the solvent. Twenty g of powdered sample of the herb was extracted by soaking in 180 mL of distilled water in a beaker, stirred for about 6 min and left overnight. Thereafter, the solution was filtered using filter paper (Whatman No. 1) and the extracts were evaporated to dryness under reduced pressure below 40°C. The plant extracts were expressed in terms of dry weight.

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In vivo neutralization assays

Lethal toxicity

The median lethal dose (LD_{50}) of *Echis carinatus* venom was determined according to the method developed by Theakston and Reid [13]. Various concentrations of venom in 0.2 ml of physiological saline was injected into the tail vein of mice (18-20 gms), 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 *Acorus calamus* and *Withania somnifera root* extracts were determined against $2LD_{50}$ of *Echis carinatus* venom. Various amount of Plant extracts were mixed with $2LD_{50}$ of venom sample and incubated at 37°C for 30 min and then injected intravenously into mice. 3–5 mice were used for each antivenom dose. Control mice received same amount of venom without antivenom (Plant extracts). The median Effective Dose (ED_{50}) was calculated from the number of deaths within 24h of injection of the venom/antivenom mixture. The ED_{50} was expressed as µl antivenom/mouse and calculated by probit analysis.

Edema- forming Activity

The Minimum edema-forming dose (MED) of *Echis carinatus* venom was determined by the method of Lomonte *et al*. and Camey *et al.* [14, 15]. Group of four mice were injected subcutaneously in the right footpad with various amounts of venom (0.25µg - 10µg) dissolved in 50 µl of phosphate–buffered saline (PBS), pH 7.2. The left footpad received 50 µl of PBS alone (control). Edema was calculated as percentage of increase in the thickness of the right foot injected with venom compared to the left foot. The thickness of each footpad was measured every 30 min after venom injection with a low-pressure spring caliper [16]. Minimum edema-forming dose (MED) was the venom dose that induced 30% edema within 6 h of venom injection when compared to control. The ability of *Acorus calamus* and *Withania somnifera* root extracts in neutralizing the edema were carried out by pre-incubating the constant amount of venom and various dilutions *Acorus calamus* and *Withania somnifera* root extracts and incubated for 30 minutes at 37°C. Then, groups of four mice (18-20 g) were injected subcutaneously in the right footpad with 50 µl 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 50 µl of PBS in the left footpad. One hour after injection, edema was evaluated as described by Yamakawa *et al.* [17]. 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 *Echis carinatus* venom was determined by the method described by Theakston and Reid [13]. The minimum haemorrhagic dose was defined as the least amount of venom which when injected intradermaly (i.d.) into mice, it resulted in a haemorrhagic lesion of 10 mm diameter in 24 h. Neutralization of the haemorrhagic activity was estimated by mixing a fixed amount of venom with various amounts *of Acorus calamus* and *Withania somnifera* root extracts. The mixture of plant extract and venom 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.

In vitro neutralization assays

Phospholipase activity

Phospholipase A_2 activity was measured using an indirect hemolytic assay on agarose–erythrocyte-egg yolk gel plate by the methods described by Gutierrez et al. [18]. Increasing concentrations of *Echis carinatus* venom was 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 $Cacl_2$. Slides were incubated at 37˚C overnight and the diameters of the hemolytic halos were measured. Control wells contained 15 µl of saline. The minimum indirect hemolytic dose (MIHD) corresponds to a concentration of venom, which produced a hemolytic halo of 11 mm diameter. The efficacy of *Acorus calamus* and *Withania somnifera* root extracts in neutralizing the phospholipase activity was carried out by mixing constant amount of venom with various amount of plant extracts and incubatig for 30 min at 37°C. Then, aliquots of the mixtures (10 µl) were added to wells in agarose-egg yolk-sheep erythrocyte gels. Control samples contained venom without Plant extracts. Plates were incubated at 37°C for 20 h. Neutralization expressed as the ratio mg antibodies/mg

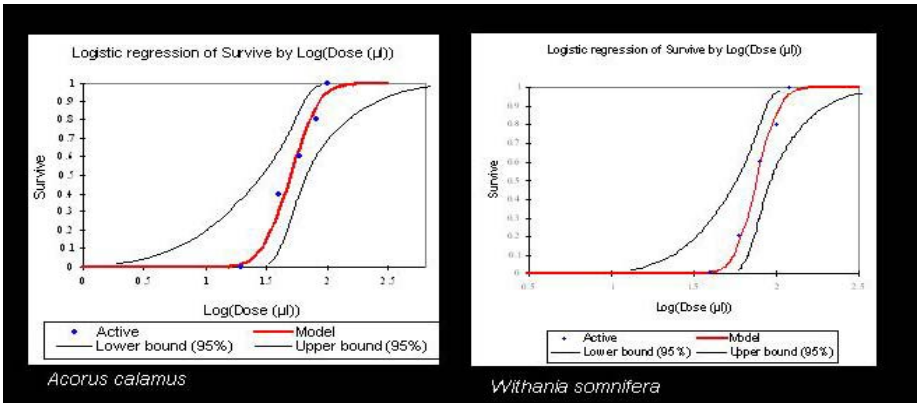

Fig 1. Dose response curve for Neutralization of Lethality by *Acorus calamus* and *Withania somnifera* root extracts against *Echis carinatus* venom in experiments involving preincubation of venom (2 x LD_{50}) and various concentrations of antivenoms (Plant extracts). The median effective dose for *Echis carinatus* venom was 0.14 mg for *Acorus calamus* and 0.16 mg for *Withania somnifera* root extracts

Table 1. Neutralization of *Echis carinatus* venom induced lethality by *Acorus calamus* and *Withania somnifera* root extracts

Plant Extracts	Concentration of *Echis carinatus* venom (µg)	Neutralization of venom by Plant extracts (ED_{50} in mg)
Acorus calamus	24 ($2LD_{50}$)	0.14 mg
Withania somnifera	24 ($2LD_{50}$)	0.16 mg

venom able to reduce 50% the diameter of the hemolytic halo when compared to the effect induced by venom alone.

Procoagulant activity

The procoagulant activity was done according to the method described by Theakston and Reid [13] modified by Laing *et al*. [19]. Various amounts of venom dissolved in 100 µl 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 concentration 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 min at 37°C. Then, 0.1 ml of mixture was added to 0.3 ml of citrated plasma and the clotting times wererecorded. 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 was 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 [16]. The minimum fibrinolytic concentration was defined as the concentration of venom that induced a fibrinolytic halo of 10 mm diameter. Neutralization experiments were performed by incubating a constant amount of venom with varying amount of *Acorus calamus* and *Withania somnifera* plant extracts at 37°C for 1 h. After incubation, the mixture was applied to the wells in the plaque. After 18 hours of incubation at 37°C, fibrinolytic halos were measured.

Statistical Analysis

Statistical evaluation was performed using XL stat 2008 and SPSS 10 Softwares. The *p values* < 0.005 was considered statistically significant.

RESULTS

Neutralization effects of *Acorus calamus* and *Withania somnifera* root extracts were tested against *Echis carinatus* venom by *in vivo* and *in vitro* methods. The lethal toxicity (LD_{50}) of *Echis carinatus* venom was assessed using Balb/c strain mice. About 12 µg of *Echis carinnatus* venom was found to be LD_{50} for mice (weight: 18 g). The neutralization of lethality was done by pre-incubating constant amount of venom with various dilutions of *Acorus calamus* and *Withania somnifera* root extracts prior to injection. We found that 0.14 mg of *Acorus calamus* and 0.16 mg of *Withania somnifera* root extracts were able to completely neutralize the lethal activity of $2LD_{50}$ of *Echis carinatus* venom (Table 1, Fig 1). In edema forming activity, 7µg of Saw-scaled viper venom induced edema within 3 h which is considered as 100% activity. The edema was reduced up to 20% when 4000 µl of plant extracts/mg venom was given. There was no further reduction in the percentage of edema even when there was an increase in anti-venom dose (Fig 2). In the case of hemorrhagic

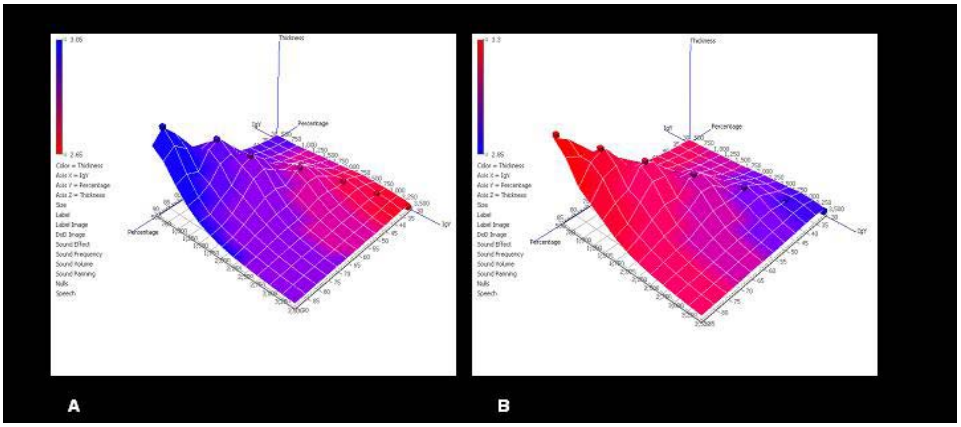

Fig 2. Neutralization of Edema induced by *Echis carinatus* venom by A) *Acorus calamus* and B) *Withania somnifera* root extracts in experiments with pre-incubation. Various mixtures of venom and antivenoms were incubated and tested in the foot pad assay. Edema was assessed 1 h after injection and expressed 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.

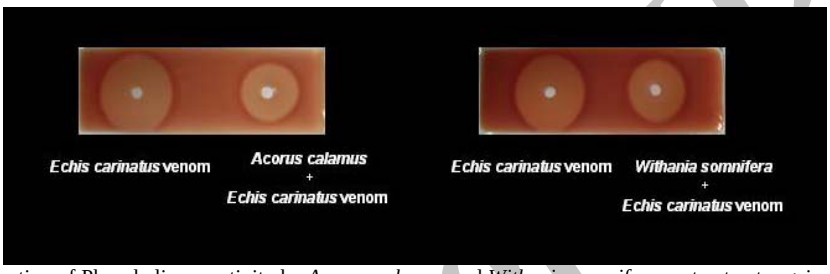

Fig 3. Neutralization of Phospholipase activity by *Acorus calamus* and *Withania somnifera* root extracts against *Echis carinatus* venom.

activity, 8 µg of venom produced a hemorrhagic spot of 10 mm diameter (MHD). Both Plant extracts were able to neutralize the hemorrhage induced by the venom. In phos pholipase activity (PLA_2), 10µg of *Echis carinatus* venom was able to produce 11-mm hemolytic halo, which is considered to be 1 U (U/10µg). *Acorus calamus* and *Withania somnifera* root extracts were capable of inhibiting PLA_2-dependent hemolysis of sheep RBC's induced by *Echis carinatus* venom in a dose-dependent manner (Table 2, Fig 3). The minimum coagulant dose (MCD) was determined and we found that 120 µg of Saw-scaled viper venom clotted human citrated plasma within 60 s. In the neutralization assay, the absence of clot formation shows the neutralizing ability of both plant extracts. High concentration of venom caused rapid clotting that required very high concentration of antivenom to neutralize. The fibrinolytic effect was effectively antagonized by the *both* plant extract. The ED_{50} of *Acorus calamus* and *Withania somnifera* root extracts against *Echis carinatus* venom were found to be 0.5 and 0.8 mg respectively.

DISCUSSION

The most efficient treatment for snake bite envenomation is the specific heterologous serum. Anti-venom against snakes bites are lacking in the rural areas of coastal region. Antiserum is the only therapeutic agent and its development from animal source is time-consuming and expensive. Although, use of plants against the effects of snakes bite has been long recognized, more scientific attention has been given for the last 20 years [20]. Many Indian medicinal plants are recommended for the treatment of snakebites [7]. In the present study, we checked the antivenom potential of *Acorus calamus* and *Withania somnifera* root extracts against *Echis carinatus* venom. It is essential to understand the pharmacological action of snake venom in order to device a rational treatment for snakebite. 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 [21]. Thus, various pharmacological activities like lethality, edema-forming activity, hemorrhagic activity, phospholipase activity (PLA_2) and pro-coagulant

Table 2. Phospholipase activity of *Echis carinatus* venom and its neutralization by *Acorus calamus* and *Withania somnifera* root extracts

Plant extracts	Dose of *Echis carinatus* venom (µg)	Neutralization of venom by plant extracts (ED_{50} in mg)
Acorus calamus	10 (1 Unit)	0.10 mg
Withania somnifera	10 (1 Unit)	0.12 mg

activity caused by *Echis carinatus* venom were carried out. Neutralization of these pharmacological effects was carried out using *Acorus calamus* and *Withania somnifera* root extracts. Neutralization studies can be performed by incubating 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 *Echis carinatus* 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. The medicinal plants *Thea sinensis Linn* and *Cordia verbenacea* were effectively neutralized the phospholipase A_2 activity induced by snake venoms [22, 23]. Edema-forming activity was assessed for *Echis carinatus* 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) concentration. Procoagulant activity induced by *Echis carinatus* venom was studied using human citrated plasma and *Acorus calamus* and *Withania somnifera* root extracts were found to be effective in the neutralization of procoagulant activity.

The present experimental results indicate that *Acorus calamus* and *Withania somnifera* root extracts were effective in neutralizing the main toxic and enzymatic effects of *Echis carinatus* venom. The antivenom properties of both plant extracts were potent enough to neutralize the lethality and various pharmacological activities of venom. The result from this preliminary study indicates that both plant extracts could be used for therapy in patients with snakebite envenomation. Further investigations are needed for identification and purification of the active components involved in the neutralization of the snake venom.

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