Pak. j. sci. ind. res. Ser. B: biol. sci. 2011 **54** (3) 157-162

Evaluation of Neutralizing Efficacy of *Acorus calamus* and *Withania somnifera* Root Extracts Against *Bangarus caeruleus* Venom

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(received February 14, 2011; revised May 19, 2011; accepted June 18, 2011)

Abstract. *Acorus calamus* and *Withania somnifera* root extracts when tested against *Bangarus caeruleus* venom, both the plant extracts neutralized various pharmacological activities induced by *B. caeruleus* venom. About 0.12 mg of *A. calamus* and 0.15 mg of *W. somnifera* root extracts completely neutralized the lethal activity of $2LD_{50}$ of *B. caeruleus* venom. Various pharmacological activities like haemorrhagic, coagulance, edematic, fibrionlytic and phospholipase activities were effectively neutralized by both the extracts.

Keywords: venoms, plant extracts, lethality, *Acorus calamus, Withania somnifera, Bangarus caeruleus*

Introduction

Snake bite constitutes major health problem in India. It is estimated that in the annual global burden of 4,21,000 envenomations and 20,000 deaths, India alone contributes 81,000 envenomations and 11,000 deaths. Snake bites are seen often among agricultural workers and among those going to the forests. Based on the above statistics, it appears that every 10 sec, one individual is envenomed and one out of four dies due to snake bite (TNHSP, 2008). The male/ female ratio among the victims is approximately 3:2. Many deaths occur before the victim reaches the hospital. Common poisonous snakes found in India are Cobra (*Naja naja*), Krait (*Bangarus caeruleus*), Russell's viper (*Daboia russelli*) and saw scaled viper (*Echis carinatus*). Antivenom immunotherapy is the only specific treatment against snake venom envenomation (Bawaskar, 2004). Antiserum development in animals is time consuming, expensive and requires ideal storage condition. There are various side effects of antivenom drugs such as anaphylactic shock, pyrogen reaction and serum sickness. Most of these symptoms may be due to the action of high concentrations of non-immunoglobulin proteins present in the commercially available horse polyvalent antivenom. 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 by traditional healers, especially in tropical areas where there are plentiful plant resources, in therapy for snake bite for a long time (Gomes *et al*., 2010; Daduang *et al*., 2005). There have been many attempts to study these plants to assess their effectiveness (Otero *et al*., 2000; Houghton and Osibogun, 1993).

India has a rich tradition of the usage of medicinal plants. Several snakebite antidotes of plant origin were recommended in old drug recipes for the treatment of snakebite (Alam and Gomes, 2003). The plants *Acorus calamus* or sweet flag (common name) is a valuable medicinal plant found almost through out India. It possesses anti-inflammatory activity, which is evident from a number of studies. *Withania somnifera* Dunal (Ashwagandha) belonging to the family Solanaceae has been used in several indigenous Ayurvedic drug preparations for maintaining health as well as for several disease conditions. In herbal medicine, ashwagandha has been traditionally used as alterative, aphrodisiac, nervine tonic, rejuvenator and in inflammation, arthritis and a number of other disorders. Methanolic extracts of *Andrographis paniculata* and *Aristolochia indica* possess potent snake venom neutralizing capacity and could potentially be used for therapeutic purposes in case of snakebite envenomation (Meenatchisundaram *et al.,* 2009a). Aqueous extract of *Mimosa pudica* root possesses compounds, which inhibit the activity of *Naja naja* and *B. caerulus* venoms (Meenatchisundaram *et al*., 2009b). The aqueous extract of *Mucuna pruriens* inhibits the activity of *N. naja* and *B. caeruleus* venoms (Meenatchisundaram *et al*., 2010). The present investigation explored the extraction and neutralizing potential of *A. calamus* and *W. somnifera* root extracts which were tested against *B. caeruleus* venom *in vivo* and *in vitro*.

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Materials and Methods

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

Medicinal plants and preparation of extracts. *A. calamus* and *W. somnifera* plants were obtained from Nehru Herbal Gardens, Coimbatore and the extracts were prepared as stated by Uhegbu *et al*. (2005) using distilled water as the solvent. 20 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 extract was evaporated to dryness under reduced pressure below 40 °C. The plant extract was expressed in terms of dry weight.

Plant lethality test. The lethal effect of plant extracts was determined by the method of Hung *et al.* (2004). Five groups of six mice each were used; each group was treated with increasing concentration of plant extract at a final volume of 0.5 mL by intravenous administration through the tail vein. LD_{50} was calculated with the confidence limit at 50% probability by the analysis of deaths occurring within 24 h of injection.

***In vivo* assessment of venom toxicity and anti-venom effect of plant extracts. *Lethal toxicity of B. caeruleus venom*.** The median lethal dose (LD_{50}) of *B. caeruleus* venom was determined according to the method developed by Theakston and Reid (1983). Various concentrations of venom in 0.2 mL of physiological saline was injected into the tail vein of mice (18-20 g), 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. calamus* and *W. somnifera root* extracts were determined against $2LD_{50}$ of *B. caeruleus* venom. Different amounts of plant extracts (µL) were mixed with $2LD_{50}$ of venom sample and incubated at 37 °C for 30 min and then injected intravenously. 3-5 mice were used for each antivenom dose. Control mice received the same amount of venom without antivenom (plant extracts). The median effective dose (ED_{50}) was calculated from the number of deaths within 24 h of injection of the venom/antivenom mixture. The ED_{50} was expressed as µL antivenom/mouse and calculated by probit analysis.

***Edema- forming activity of B. caeruleus venom*.** Minimum edema-forming dose (MED) of *B. caeruleus* venom was determined by the method of Lomonte *et al*. (1993). Group of four mice were injected sub-cutaneously in the right footpad with various amounts of venom (0.25 µg-10 µg) 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 (Rojas *et al*., 2005). 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 *A. calamus* and *W. somnifera* root extracts in neutralizing the edema-forming activity was determined by pre-incubating constant amount of venom and various dilutions of *A. calamus* and *W. somnifera* root extracts and incubating for 30 min at 37 °C. Then, groups of four mice (18-20 g) were injected sub-cutaneously in the right footpad with 50 µL of the mixtures, containing venom/plant extracts, whereas the left footpad received 50 µL of PBS alone. Control mice were injected with venom in the right footpad and 50 µL of PBS in the left footpad. Edema evaluated one hour after injection as described by Yamakawa *et al.* (1976) and was expressed as the percentage increase in thickness of the right footpad compared to the right footpad of the control mice.

***Haemorrhagic activity*.** Minimum haemorrhagic dose (MHD) of *B. caeruleus* venom was determined by the described method of Theakston and Reid (1983). 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 10 mm diameter in 24 h. Neutralization of the haemorrhagic activity was estimated by mixing a fixed amount of venom with different amounts of *A. calamus* and *W. somnifera* root 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.

***In vitro* assessment of venom toxicity and anti-venom efficacy. *Phospholipase activity*.** Phospholipase A2 activity was measured using an indirect haemolytic

assay on agarose-erythrocyte-egg yolk gel plate by the method described by Gutierrez *et al*. (1988). Increasing concentrations of *B. caeruleus* venom (µg) 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 haemolytic halos were measured. Control wells contained 15 µL of saline. The minimum indirect haemolytic dose (MIHD) corresponds to a concentration of venom, which produces a haemolytic halo of 11 mm diameter. The efficacy of *A. calamus* and *W. somnifera* root extracts in neutralizing the phospholipase activity was assessed by mixing the same amount of venom (µg) with different amounts of plant extracts (µL) and incubated for 30 min at 37 °C. Then, aliquots of 10 µL of the mixtures were added to wells in agarose-egg yolk-sheep erythrocyte gels. Control samples contained venom without plant extract. Plates were incubated at 37 °C for 20 h. Neutralization was expressed as the ratio mg antibodies/mg venom able to reduce by 50% the diameter of the haemolytic halo as compared to the effect induced by venom alone.

***Procoagulant activity*.** The procoagulant activity was assessed according to the method described by Theakston and Reid (1983) modified by Laing *et al*. (1992). 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 sec. 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 the mixture was added to 0.3 mL of citrated plasma and the clotting time recorded. In control tubes, plasma was incubated with either venom alone or plant extract 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 as compared to the clotting time of plasma incubated with two MCD of venom alone.

***Fibrinolytic activity*:** A modified plaque assay was used (Laing *et al*., 1992). 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 constant amount of venom with varying amounts of *A. 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 h of incubation at 37 °C, fibrinolytic halos were measured.

Double immunodiffusion. The interaction of *A. calamus* and *W. somnifera root* extracts against *B. caeruleus* venom was evaluated using double immunodiffusion technique (Ouchterlony, 1953). Agar (1 g) was boiled in 100 mL water in microwave oven. The agar solution was then allowed to cool to 45 °C and then 3.5 mL of it was pipetted onto a precoated slide. The slide was left to solidify at room temperature after which four holes were punched out using a gel puncher. The plugs of agar were removed from each well with a Pasteur pipette attached to a vacuum line. The test was conducted by pipetting 20 µL of *B. caeruleus* venom (10 mg/mL) and 20 µL of crude plant extract (100 mg/mL) into wells 1 and 2, respectively, followed by incubation overnight at room temperature in a humid chamber. The slide was examined for precipitation lines.

Statistical analysis. Statistical evaluation was performed using XL stat 2008 and SPSS 10 Softwares. $P < 0.005$ was considered statistically significant.

Results and Discussion

The lethal toxicity (LD_{50}) of the venom and of the two root extracts was assessed using 18 g, Balb/c strain mice. About 3 µg of the venom was found to be LD_{50} for 18 g mice. About 1.2 mg and 1.4 mg was found to be the lethal toxicity (LD_{50}) for *A. calamus* and *W. somnifera* root extracts, respectively. Neutralization of lethality was carried out by preincubating constant amount of venom with various dilutions of the two root extracts prior to injection. It was found that 0.12 mg of *A. calamus* and 0.15 mg of *W. somnifera* root extracts were able to completely neutralize the lethal activity of $2LD_{50}$ of the *B. caeruleus* venom (Table 1, Fig. 1). In edema forming activity, 2 µg of the *venom* induced edema formation within 3 h which is considered as 100% activity. The edema was reduced down 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 antivenom dose (Fig. 2). In the case of haemorrhagic activity, 2 µg of venom produced a haemorrhagic spot of 10 mm diameter (MHD). Both plant extracts were able to neutralize the haemorrhage induced by the venom. In phospholipase activity (PLA2), 10 µg of *B. caeruleus* venom was able to produce 11 mm diameter haemolytic

halo, which is considered to be 1U (U/10 µg). Both root extracts were capable of inhibiting PLA2 dependent haemolysis of sheep RBC's induced by the venom in a dose dependent manner (Table 2). Minimum coagulant dose (MCD) was determined and it was found that 40 µg of the viper venom clotted 0.3 mL human citrated plasma within 60 sec. In the neutralization assay, the absence of clot formation shows the neutralizing ability of both the plant extracts. High concentration of venom caused rapid clotting that required very high concentration of anti-venom to neutralize. The fibrinolytic effect was effectively antagonized by both the plant extract. The ED_{50} of *A. calamus* and *W. somnifera* root extracts against the venom were found to be 0.4 and 0.7 mg, respectively. In Double Immunodiffusion test, a visible precipitin line was form between the venom and the plant extracts showing that plant extracts possess potent snake venom neutralizing compounds.

Table 1. Neutralization of *B. caeruleus* venom induced lethality by *A. calamus* and *W. somnifera* root extracts

Plant extracts	Concentration of *B. caeruleus* venom (µg)	Neutralization of venom by plant extracts (ED_{50} in mg)
Acorus calamus	6 ($2LD_{50}$)	0.12 mg
Withania somnifera	6 ($2LD_{50}$)	0.15 mg

Table 2. Phospholipase activity of *B. caeruleus* venom and its neutralization by *A. calamus* and *W. somnifera* root extracts

Plant extracts	Dose of *B. caeruleus* venom (µg)	Neutralization of venom by plant extracts (ED_{50} in mg)
A. calamus	10 (1 Unit)	0.11 mg
W. somnifera	10 (1 Unit)	0.14 mg

Snakebites are encountered all over the country with a rural/urban ratio of 9:1. They are more common during monsoon and post monsoon seasons. Many of the susceptible populations are poor living below poverty line, living in rural areas with less access to health care. About 10% of snakebite deaths are among the victims who come to the hospital and about 90% die outside, having under-gone other remedies like *mantra*, magic, and so on. The hospital stay varies from 2 to 30 days, the median being 4 days. The in-hospital mortality varies from 5 to 10%, and the causes are acute renal failure, respiratory failure, sepsis, bleeding and others (TNHSP, 2008). Antivenom against snakes bites are lacking in the rural areas of coastal region. The most common and effective method of treating snake bite victims is through administration of antivenom, a serum made from the venom of the snake (Gomes *et al*., 2010). Although, use of plants against the effects of snakes bite has been long recognized, more scientific attention has been given during the last 20 years. In our present study we assessed the antivenom potential of *A. calamus* and *W. somnifera* root extracts against *B. caeruleus* 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

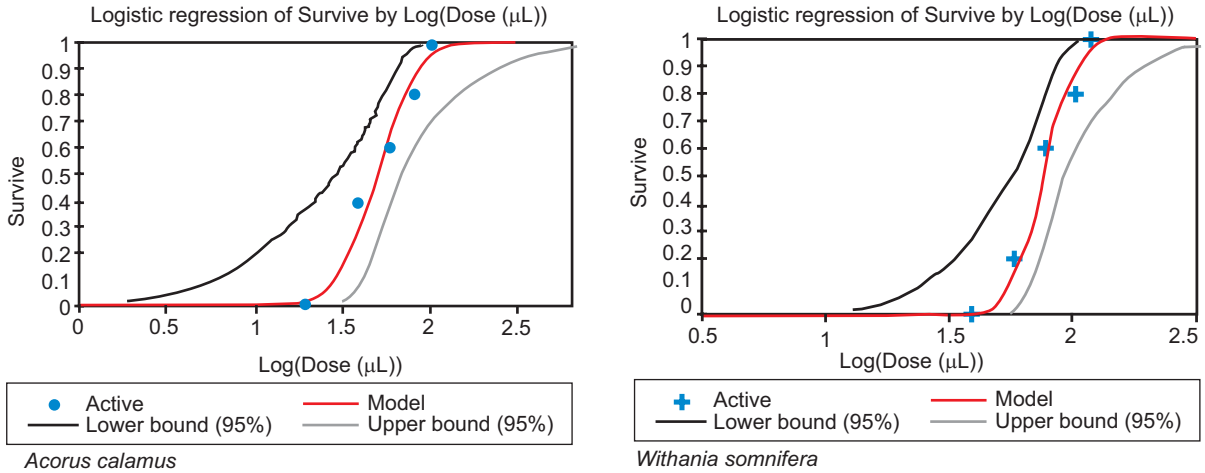

Fig. 1. Dose response curve for neutralization of lethality by *A. calamus* and *W. somnifera* root extracts against *B. caeruleus* venom.

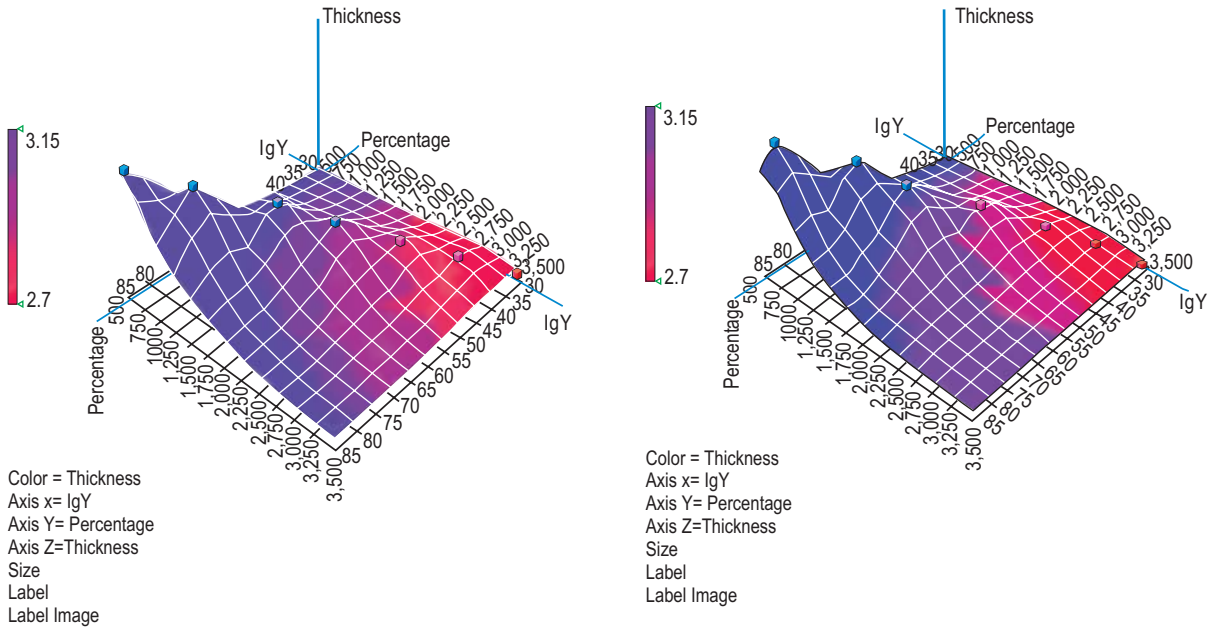

Fig. 2. Neutralization of edema induced by *B. caeruleus* venom by (A) *A. calamus* and (B) *W. somnifera* root extracts in experiments with pre-incubation. Results presented as mean ± SE (N=3). P<0.005 at all antivenoms/venom ratios.

(WHO, 2004). Herbal compounds that possess anti-snake venom activity are tested in experimental animal models through administering in different ways: (A) venom-herbal compounds mixed together, (B) herbal compounds followed by venom and (C) venom followed by herbal compounds (Gomes *et al*., 2010). In the present preliminary work the anti venom properties of plant extracts were tested by pre-incubation method venom-herbal compounds mixed together and tests for various pharmacological activities like lethality, edema forming activity, hemorrhagic activity, phospholipase activity (PLA2), procoagulant activity caused by *B. caeruleus* venom were undertaken. Neutralization studies can be performed by incubating of venom and plant extracts prior to testing (pre-incubation method). The results showed that both the plant extracts were capable of neutralizing the lethality induced by the venom. The venom showed the presence of PLA2 enzymes through producing haemolytic haloes in indirect haemolytic assays. Both the plant extracts were capable of inhibiting PLA2 dependent haemolysis of sheep RBCs in a dose dependent manner. The medicinal plants, *Camellia sinensis* L. and *Cordia verbenacea* effectively neutralized the phospholipase A2 activity induced by snake venoms (Ticli *et al*., 2005; Fattepur and Gawad, 2004). Edema-forming activity was assessed for *B. caeruleus* venom and both plant extracts were found to be effective in neutralization of edema induced by venom. 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 the venom was studied using human citrated plasma and both the root extracts were found to be effective in the neutralization of procoagulant activity. The present experimental results indicate that *A. calamus* and *W. somnifera* root extracts were effective in neutralizing the main toxic and enzymatic effects of *B. caeruleus* venom. The anti-venom properties of both the plant extracts were potent enough to neutralize the lethality and various pharmacological activities of venom. The result from this preliminary study indicates that both the plant extracts have the potential to 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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