

RESEARCH ARTICLE



# 2 In Vivo and In Vitro Studies on Neutralizing Effects of Acorus calamus and Withania somnifera root aextracts against Echis carinatus venom

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# **ABSTRACT**

10 Neutralization effects of Acorus calamus and Withania somnifera root extracts were tested against Echis a carinatus venom. Both plant extracts were effectively neutralized the various pharmacological activities 12 induced by Echis carinatus venom. About 0.14 mg of Acorus calamus and 0.16 mg of Withania somnifera 13 root extracts were able to completely neutralize the lethal activity of 2LD<sub>50</sub> of *Echis carinatus* venom. 14 Various pharmacological activities like haemorrhagic, coagulant, edema and phospholipase activities 15 were effectively neutralized by both plant extracts. The above observations confirmed that both plant 16 extracts possess potent snake venom neutralizing compounds, which inhibit the activity of Echis carinatus 17 venoms.

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

20 particularly among the forest workers and agriculturists 47 extracts possess potent snake venom neutralizing 21 in rural India. There are over 2000 species of snakes in 48 capacity and could potentially be used for therapeutic 22the world and about 216 species exists in India, of 49purposes in case of snakebite envenomation [8]. 23 which 52 are Venomous [1]. The common poisonous 50 Aqueous extract of Mimusa pudica root possesses 24snakes found in India are Cobra (Naja naja), Krait 51compounds, which inhibit the activity of Naja naja and 25(Bangarus caeruleus), Russell's viper (Daboia russelli) 52Bangarus caerulus venoms. He also reported that 26 and Saw Scaled Viper (*Echis Carinatus*) [2]. In India, 53 aqueous extracts of *M. pudica* root possess compounds, 27 about 15,000 persons are affected every year by snake 54 which inhibit the activity of Russell's viper and Saw 28 envenomation. Echis carinatus (Saw-scaled viper) is 55 scaled viper venoms [9, 10]. Aqueous extracts of 29 responsible for a large number of snake bite case, 56 Mucuna pruriens seeds possess compounds, which 30 reaching 95% of envenomations in the state of Jammu 57 inhibit the activity of cobra and krait venoms. [11]. The 31[3]. Antivenom immunotherapy is the only specific 58 present investigation explored the Neutralization effects 32 treatment against snake venom envenomation.

34 consuming, expensive and requires ideal storage 61 methods. 35 condition. Over the years, many attempts have been 36 made for the development of snake venom antagonists 37 especially from plants sources. Extracts from plants 62 38 have been used among traditional healers, especially in 39tropical areas where there are plentiful sources for 40 snakebite therapy for a long time [4]. In modern 64 41 science, there have been many attempts to study these 65 carinatus was obtained from Irula's Snake Catchers 42plants to clarify their effectiveness [5, 6]. India has a 66Industrial Co-operative Society Limited, Chennai and 43rich tradition of the usage of medicinal plants. Many 67was stored at 4°C. Male inbreed Swiss albino mice (18-44Indian medicinal plants are recommended for the 6820 gm) were used for the studies of venom toxicity and

Snake bite is a serious medico-legal problem 46Andrographis paniculata and Aristolochia indica plant 59 of Acorus calamus and Withania somnifera root extracts Antiserum development in animal is time- 60 against Echis carinatus venom using in vivo and in vitro

# **MATERIALS AND METHODS**

# 63 Venom and Experimental animals

The free-dried snake venom powder of Echis 45 treatment of snakebite [7]. Methanolic extracts of 69 in the experiments of venom neutralization. Institutional

### A. calamus and W. somnifera Effects on Echis carinatus venom

70 Animal Ethics Committee clearance at Institute of 125 dose that induced 30% edema within 6 h of venom 71 vector control and Zoonooses, Hosur, was obtained to 126 injection when compared to control. The ability of 72 conduct the experiment.

### 73 Medicinal Plants and Preparation of Extracts

75 obtained from Nehru Herbal Gardens, Coimbatore and 76 the extracts were prepared by the method of Uhegbu et 77 al. [12] using distilled water as the solvent. Twenty g of 133 subcutaneously in the right footpad with 50 µl of the 78 powdered sample of the herb was extracted by soaking<sup>134</sup> mixtures, containing venom/plant extracts, whereas the 79 in 180 mL of distilled water in a beaker, stirred for<sup>135</sup>left footpad received 50µl of PBS alone. Control mice 80 about 6 min and left overnight. Thereafter, the solution 136 were injected with venom in the right footpad and 50 µl 81 was filtered using filter paper (Whatman No. 1) and the 137 of PBS in the left footpad. One hour after injection, 82extracts were evaporated to dryness under reduced<sup>138</sup>edema was evaluated as described by Yamakawa et al. <sup>83</sup>pressure below 40°C. The plant extracts were expressed<sup>139</sup>[17]. Edema was expressed as the percentage increase in 84 in terms of dry weight.

85 Neutralization effects of Acorus calamus and 86 Withania somnifera root extracts against Echis 87 carinatus venom

### 88 In vivo neutralization assays

### 89 Lethal toxicity

The median lethal dose  $(LD_{50})$  of *Echis carinatus* 91 venom was determined according to the method 92 developed by Theakston and Reid [13]. Various 93 concentrations of venom in 0.2 ml of physiological 94 saline was injected into the tail vein of mice (18-20 95 gms), using groups of 3-5 mice for each venom dose. 96 The LD<sub>50</sub> was calculated with the confidence limit at 9750% probability by the analysis of deaths occurring 98 within 24 h of venom injection. The anti-lethal 99 potentials of Acorus calamus and Withania somnifera 155 mice. The haemorrhagic lesion was estimated after 24 h. 100 root extracts were determined against 2LD<sub>50</sub> of Echis

101 carinatus venom. Various amount of Plant extracts were 156 In vitro neutralization assays 102 mixed with 2LD<sub>50</sub> of venom sample and incubated at 157 Phospholipase activity 10337°C for 30 min and then injected intravenously into 104 mice. 3–5 mice were used for each antivenom dose.<sup>158</sup> 105Control mice received same amount of venom without 159 indirect hemolytic assay on agarose-erythrocyte-egg 106 antivenom (Plant extracts). The median Effective Dose 160 yolk gel plate by the methods described by Gutierrez et 107 (ED<sub>50)</sub> was calculated from the number of deaths within 161 al. [18]. Increasing concentrations of *Echis carinatus* 10824h of injection of the venom/antivenom mixture. The162 venom was added to 3-mm wells in agarose gels (0.8% 109ED<sub>50</sub> was expressed as µl antivenom/mouse and 163 in PBS, pH 8.1) containing 1.2% sheep erythrocytes, 110 calculated by probit analysis.

### 111 Edema- forming Activity

The Minimum edema-forming dose (MED) of Echis<sub>167</sub>Control wells contained 15 µl of saline. The minimum 113 carinatus venom was determined by the method of 168 indirect hemolytic dose (MIHD) corresponds to a 114Lomonte et al. and Camey et al. [14, 15]. Group of four 169concentration of venom, which produced a hemolytic 115 mice were injected subcutaneously in the right footpad 170 halo of 11 mm diameter. The efficacy of Acorus 116 with various amounts of venom (0.25µg - 10µg)171 calamus and Withania somnifera root extracts in 117 dissolved in 50 µl of phosphate-buffered saline (PBS),172 neutralizing the phospholipase activity was carried out 118pH 7.2. The left footpad received 50 µl of PBS alone173by mixing constant amount of venom with various 119(control). Edema was calculated as percentage of 174 amount of plant extracts and incubatig for 30 min at 120 increase in the thickness of the right foot injected with 17537°C. Then, aliquots of the mixtures (10 µl) were added 121 venom compared to the left foot. The thickness of each176 to wells in agarose-egg yolk-sheep erythrocyte gels. 122 footpad was measured every 30 min after venom177 Control samples contained venom without Plant 123 injection with a low-pressure spring caliper [16].178 extracts. Plates were incubated at 37°C for 20 h. 124 Minimum edema-forming dose (MED) was the venom179 Neutralization expressed as the ratio mg antibodies/mg

127Acorus calamus and Withania somnifera root extracts in 128 neutralizing the edema were carried out by pre-129 incubating the constant amount of venom and various Acorus calamus and Withania somnifera plants were 130 dilutions Acorus calamus and Withania somnifera root 31 extracts and incubated for 30 minutes at 37°C. Then, 32 groups of four mice (18-20 g) were injected 140 thickness of the right footpad compared to the right 141 footpad of the control mice.

### 142 Haemorrhagic activity

The minimum haemorrhagic dose (MHD) of Echis 144 carinatus venom was determined by the method 145 described by Theakston and Reid [13]. The minimum 146 haemorrhagic dose was defined as the least amount of 47 venom which when injected intradermaly (i.d.) into 48 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 40.1 ml of the mixture was injected intradermaly into

Phospholipase A2 activity was measured using an 1641.2% egg yolk as a source of lecithin and 10 mM Cacl<sub>2</sub>. 165Slides were incubated at 37°C overnight and the 166 diameters of the hemolytic halos were measured.



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 LD50) 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 <i>Echis carinatus</i> venom (µg)	Neutralization of venom by Plant extracts (ED <sub>50</sub> in mg)
Acorus calamus	24 (2LD <sub>50</sub> )	0.14 mg
Withania somnifera	24 (2LD <sub>50</sub> )	0.16 mg

180 venom able to reduce 50% the diameter of the 210 with varying amount of Acorus calamus and Withania 181 hemolytic halo when compared to the effect induced by 182 venom alone.

## 183 Procoagulant activity

The procoagulant activity was done according to the 215 Statistical Analysis 185 method described by Theakston and Reid [13] modified 186 by Laing et al. [19]. Various amounts of venom<sup>216</sup> 187 dissolved in 100 µl PBS (pH 7.2) was added to human<sup>2</sup> 188 citrated plasma at 37°C. Coagulation time was recorded<sup>2</sup> 189 and the minimum coagulant dose (MCD) was 190 determined as the venom concentration which induced 219 191 clotting of plasma within 60 seconds. Plasma incubated 192 with PBS alone served as control. In neutralization 193 assays, constant amount of venom was mixed with 194 various dilutions of plant extracts. The mixtures were 195 incubated for 30 min at 37°C. Then, 0.1 ml of mixture 196 was added to 0.3 ml of citrated plasma and the clotting 197 times wererecorded. In control tubes, plasma was 198incubated with either venom alone or plant extracts 199alone. Neutralization was expressed as effective dose228 various dilutions of Acorus calamus and Withania 200(ED), defined as the ratio µl antivenom (plant<sub>229</sub> somnifera root extracts prior to injection. We found that 201 extracts)/mg venom at which the clotting time was2300.14 mg of Acorus calamus and 0.16 mg of Withania 202 increased three times when compared with clotting time 231 somnifera root extracts were able to completely 203 of plasma incubated with two MCD of venom alone.

### 204 Fibrinolytic activity

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, 214 fibrinolytic halos were measured.

Statistical evaluation was performed using XL stat 172008 and SPSS 10 Softwares. The *p* values < 0.005 was 18 considered statistically significant.

### RESULTS

Neutralization effects of Acorus calamus and Withania somnifera root extracts were tested against 2*Echis carinatus* venom by *in vivo* and *in vitro* methods. The lethal toxicity (LD<sub>50</sub>) of *Echis carinatus* venom was 4 assessed using Balb/c strain mice. About 12 µg of Echis *carinnatus* venom was found to be LD<sub>50</sub> for mice (weight: 18 g). The neutralization of lethality was done 7 by pre-incubating constant amount of venom with 232 neutralize the lethal activity of 2LD<sub>50</sub> of *Echis carinatus* 233venom (Table 1, Fig 1). In edema forming activity, 7µg 234 of Saw-scaled viper venom induced edema within 3 h

A modified plaque assay was used [16]. The 235 which is considered as 100% activity. The edema was 206 minimum fibrinolytic concentration was defined as the 236 reduced up to 20% when 4000 µl of plant extracts/mg 207 concentration of venom that induced a fibrinolytic halo237 venom was given. There was no further reduction in the 208 of 10 mm diameter. Neutralization experiments were 238 percentage of edema even when there was an increase in 209 performed by incubating a constant amount of venom239 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  $\pm$  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. 

240 activity, 8 µg of venom produced a hemorrhagic spot of 203 24110 mm diameter (MHD). Both Plant extracts were able 242to neutralize the hemorrhage induced by the venom.

245 venom was able to produce 11-mm hemolytic halo, 268 agent and its development from animal source is time-246 which is considered to be 1 U (U/10µg). Acorus 247 calamus and Withania somnifera root extracts were 248 capable of inhibiting PLA<sub>2</sub>-dependent hemolysis of  $^{270}_{271}$  recognized, more scientific attention has been given for 249 sheep RBC's induced by *Echis carinatus* venom in a 272 the last 20 years [20]. Many Indian medicinal plants are 250 dose-dependent manner (Table 2, Fig 3). The minimum<sub>273</sub> recommended for the treatment of snakebites [7]. In the 251 coagulant dose (MCD) was determined and we found<sub>274</sub> present study, we checked the antivenom potential of 252 that 120 µg of Saw-scaled viper venom clotted human<sub>275</sub>Acorus calamus and Withania somnifera root extracts 253 citrated plasma within 60 s. In the neutralization assay, 276 against *Echis carinatus* venom. It is essential to 254 the absence of clot formation shows the neutralizing277 understand the pharmacological action of snake venom 255 ability of both plant extracts. High concentration of 278 in order to device a rational treatment for snakebite. The 256 venom caused rapid clotting that required very high279 neutralization ability of snake antivenoms is still 257 concentration of antivenom to neutralize. The 280 assessed by the traditional in vivo lethality assay 258 fibrinolytic effect was effectively antagonized by the 281 (minimum effective dose ED<sub>50</sub>), comparable to those 259 both plant extract. The ED<sub>50</sub> of Acorus calamus and 282 used for bacterial antitoxins, usually performed in mice 260 Withania somnifera root extracts against Echis<sub>283</sub>[21]. Thus, various pharmacological activities like 261 carinatus venom were found to be 0.5 and 0.8 mg284 lethality, edema-forming activity, hemorrhagic activity, 262 respectively.

### DISCUSSION

The most efficient treatment for snake bite 265 envenomation is the specific heterologous serum. Anti-

244 pholipase activity (PLA<sub>2</sub>), 10µg of *Echis carinatus*<sup>266</sup> venom against snakes bites are lacking in the rural areas 269 consuming and expensive. Although, use of plants 270 against the effects of snakes bite has been long 285 phospholipase activity (PLA2) and pro-coagulant

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

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

286 activity caused by *Echis carinatus* venom were carried<sup>3488</sup>.  $^{287}$ out. Neutralization of these pharmacological effects was $^{349}_{350}$ 288 carried out using Acorus calamus and Withania 350 289 somnifera root extracts. Neutralization studies can be 3529 290 performed by incubating venom and plant extracts prior<sub>353</sub> 291 to testing (pre-incubation method). The results showed 354 292that the both plant extracts were capable of neutralizing<sup>355</sup> 293 the lethality induced by the venom. The *Echis carinatus*<sup>35610</sup>. 294 venom showed the presence of  $PLA_2$  enzymes by means<sup>3</sup> 295 of producing hemolytic haloes in indirect hemolytic 296 assays. Both plant extracts were capable of inhibiting  $^{359}_{360}$ 5911 297 PLA<sub>2</sub>-dependent hemolysis of sheep RBCs in a dose- $\frac{361}{361}$ 298 dependent manner. The medicinal plants Thea sinensis 36212. 299Linn and Cordia verbenacea were effectively 363 300 neutralized the phospholipase A2 activity induced by 364 301 snake venoms [22, 23]. Edema-forming activity was<sup>365</sup> 302 assessed for *Echis carinatus* venom and both plant<sup>366</sup>13. 303 extracts were found to be effective in neutralization of 367 304edema induced by venoms. There was a significant <sup>368</sup>  $_{305}^{36914}$  decrease in the edema (footpad thickness) when there  $_{370}^{36914}$ . 306 was an increase in the antivenom (plant extract)371307 concentration. Procoagulant activity induced by Echis372 308 carinatus venom was studied using human citrated 37315. 309 plasma and Acorus calamus and Withania somnifera 374 310root extracts were found to be effective in the 375 311 neutralization of procoagulant activity.

The present experimental results indicate that 37716. 313Acorus calamus and Withania somnifera root extracts 314 were effective in neutralizing the main toxic and 315 enzymatic effects of *Echis carinatus* venom. The 17. 316 antivenom properties of both plant extracts were potent 317 enough to neutralize the lethality and various <sup>318</sup>pharmacological activities of venom. The result from<sup>3</sup> 319 this preliminary study indicates that both plant extracts 386 320 could be used for therapy in patients with snakebite<sub>387</sub> 321 envenomation. Further investigations are needed for 388 322identification and purification of the active components 38919. 323 involved in the neutralization of the snake venom.

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