Neutralization potential of *Viper russelli russelli* (Russell’s viper) venom by ethanol leaf extract of *Acalypha indica*

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Received 22 September 2003; received in revised form 18 April 2004; accepted 7 May 2004

Available online 28 July 2004

Abstract

The study aims to examine the *Viper russelli russelli* venom neutralization potential of the ethanol leaf extract (250, 500 and 750 mg/kg) of *Acalypha indica* (Euphorbiaceae). Administration of the ethanol leaf extract at i.p. dose levels of 500 and 750 mg/kg significantly inhibited, in a dose dependent manner, the *Viper russelli* venom-induced lethality, haemorrhage, necrotizing and mast cell degranulation in rats and the cardiotoxic and neurotoxic effects in isolated frog tissue. Administration of the extract also significantly inhibited venom-induced lipid peroxidation in RBC, decreased GSH and catalase levels of rat kidney tissue. The observations confirmed that the ethanol leaf extract of *Acalypha indica* possesses potent snake venom neutralizing properties.

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Keywords: *Viper russelli russelli*, *Acalypha indica*, Snake venom neutralization

1. Introduction

Snake envenomation is a WHO identified occupational hazard for paddy farmers in South East Asian countries. Incidents of snakebite leading to death are common in many tropical countries during or after the rainy season because of increased human settlements in the natural habitats of snakes. Russell’s viper or daboia, *Viper russelli* appears to be the commonest cause of fatal snakebite in Southern India (De Silva and Ranasinghe, 1983). *Viper russelli russelli* (Indian subspecies of Russell’s viper) is one of the four major classes of snakes that cause death in the Indian sub-continent. Anti serum, the only remedy for envenomation, may be associated with various reactions viz, early anaphylactoid reaction, pyrogenic reaction and late serum sickness reaction, besides several other manifestations (Singh et al., 2001). In addition anti serum development in animals (Horse, Goat, Sheep etc) is time consuming, expensive and requires ideal storage conditions (Bawaskar and Bawaskar, 2001). Many Indian medicinal plants have been recommended for the treatment of snakebite (Chopra et al., 1956). *Acalypha indica* Linn. (family: Euphorbiaceae), known in Telugu as Kuppichettu or Harita Manjiri, in Tamil as Kuppi meni and in Kannada as Kuppiguda, is a small annual shrub which generally occurs as a troublesome weed in gardens, roadsides and throughout the plains of India (Chopra et al., 1956). The plant is used as an antidote (Houghton et al., 1993) for snakebite in the Hardoi District of Northern India (Siddiqui and Husain, 1990), by the Nakkala tribe of Chittor and in East Godavari Districts of Andhra Pradesh (Suryanarayana, 1996). The present study aims to examine the venom neutralization potential of ethanol leaf extract of *Acalypha indica*.

2. Methodology

2.1. Venom

The lyophilised snake venom *Viper russelli* was obtained from Irla Snake Catcher’s I.C.S. Ltd., Vadananmelli Village, Kancheepuram District, Tamil Nadu, India, and was preserved at 4°C. Before use, the venom was dissolved in saline, centrifuged at 2000rpm for 10 min and the supernatant was used for anti venom studies. Venom concentration was expressed in terms of dry weight.

2.2. Plant material

The leaves of *Acalypha indica* (family: Euphorbiaceae) were collected from Khammam District, Andhra Pradesh,
under standard conditions (12 h light and 12 h dark cycle; 25 ± 30 °C; 35–60% humidity), the animals were fed with standard rat pellet diet (Hindustan Lever Ltd., Mumbai, India) and water ad libitum. The study was approved by the Institutional Animal Ethical Committee of KMC, Manipal, India (IAEC/KMC/2002–2003).

2.3. Animals

Healthy adult Wistar albino rats weighing about 200–250 g and Swiss albino mice weighing about 20–25 g between 2 and 3 months of age were used for the study. Housed individually in polypropylene cages, maintained under standard conditions (12 h light and 12 h dark cycle; 25 ± 30 °C; 35–60% humidity), the animals were fed with standard rat pellet diet (Hindustan Lever Ltd., Mumbai, India) and water ad libitum. The study was approved by the Institutional Animal Ethical Committee of KMC, Manipal, India (IAEC/KMC/2002–2003).

2.4. Preparation of ethanol extract

The shade dried coarsely powdered leaves (1 kg) were subjected to Soxhlet extraction using ethanol (95%). The solvent was removed in vacuo and the extract was used for chemical and pharmacological studies.

2.5. Phytochemical screening

Preliminary phytochemical screening carried out according to the procedure given by Harborne (1984), revealed the presence of steroids, carbohydrates, flavonoids and alkaloids.

2.6. Snake venom anti serum

Lyophilised polyvalent snake venom anti serum (as reference serum) was obtained from Vins Bioproducts Ltd., Eradur 502 329, Medak District, A.P., India. Before use the anti serum was dissolved in 10 ml of water for injection.

2.7. Neutralization of lethal venom effect

The toxicity of Viper russelli venom was assessed by i.p. administration of different concentrations of venom dissolved in 0.2 ml of physiological saline to groups (n = 8) of Swiss albino mice (18–20 g). The median lethal dose (LD50) of venom was determined 24 h later by the method of Theakston and Reid (1983). The neutralizing potency of the leaf extract was assessed by i.p. administration of LD50 dose of venom into groups of mice (n = 8), followed by i.p. administration of different doses of the plant extract. The standard reference group was administered snake venom anti serum after administration of LD50 dose of venom.

2.8. Neutralization of haemorrhagic activity

The minimum haemorrhagic dose (MHD), i.e. the least amount of venom (μg/dry weight) which when injected intradermally into rats results in a haemorrhagic lesion of 10-mm diameter 24 h later, was determined by the method of Kondo (1960). The MHD dose, intradermally injected into the shaved dorsal skin of the rats was followed after 5 min by i.p. administration of the different doses of plant extract.

2.9. Neutralization of necrotizing activity

The minimum necrotizing dose (MND), i.e. the least amount of venom (μg/dry weight) which when injected intradermally into rats results in a necrotic lesion of 5-mm diameter 3 days later, was determined by the method of Theakston and Reid (1983). The MND dose of venom, intradermally injected into the shaved dorsal skin of the rats was followed after 5 min by i.p. administration of different doses of the plant extract.

2.10. Neutralization of cardiotoxic activity

The effect of the ethanol leaf extract of Acalypha indica on Viper russelli induced changes in isolated frog heart was determined by the method of Kannappa Reddy et al. (1993). The isolated frog was connected to the perfusion apparatus containing Ringer solution and the flow rate maintained between 5 and 7 ml/min. Graded doses of the ethanol leaf extract and viper venom (1–4 μg) were injected and the changes in contraction studied. The effect of Viper russelli venom in the presence of the leaf extract was also studied. The experiment was repeated in five isolated preparations.

2.11. Neutralization of neurotoxic activity

Effect of ethanol leaf extract of Acalypha indica on Viper russelli induced changes on skeletal muscle was determined by the method of Kannappa Reddy et al. (1993). The rectus abdominus muscle isolated from a pithed frog was mounted in an organ bath of 30-ml capacity, containing Ringer solution. The effect of acetylcholine, ethanol leaf extract and Viper russelli venom per se and the effect of acetylcholine and Viper russelli venom in the presence of leaf extract was recorded. The experiment was repeated in five isolated preparations.

2.12. Neutralization of mast cell degranulation activity

The in vitro method of Kannappa Reddy et al. (1993) was modified for in vivo determination of mast cell degranulation. The LD50 dose of Viper russelli venom injected (i.p.) to groups of rats, were followed after 5 min by administration of different i.p. doses of leaf extract. After 24 h, the rats were sacrificed and mesentery cut into small bits of about 1 cm. These were carefully washed with tyrode solution and...
spread over glass slides. The mast cells, stained with 1% toluidine blue were counted under a high power objective field and the percentage of degranulation was noted.

2.13. Biochemical estimation

Viper russelli (LD50) venom, administered (i.p.) into groups of rats was followed after 5 min by i.p. administration of different doses of ethanol leaf extract. Reference standard snake venom anti serum was administered to one group of animals. After 24 h, blood samples collected through the retro orbital sinus of the different groups were collected in bottles containing EDTA (1 µg/ml). The blood was used for the estimation of lipid peroxidation. Rats were sacrificed and their kidneys isolated for estimation of GSH and catalase.

2.13.1. RBC preparation

Blood was centrifuged at 3000 rpm for 10 min. Plasma was separated and stored at 4–5 °C till further use. Erythrocytes were washed thrice with 0.01 mol/l saline phosphate buffer, pH 7.4, then diluted 1:2 with the same buffer and stored at 4–5 °C for further use.

2.13.1.1. Estimation of lipid peroxidation. The assay for lipid peroxidation in RBC was determined by measuring the amount of thiobarbituric acid reactive substances (TBARS) formed, following the method of Kartha and Krishnamurthy (1978). Packed cells (0.2 ml) were mixed with 0.835 ml phosphate buffer saline and 0.5 ml trichloroacetic acid (TCA). The reaction mixture was incubated at 4 °C for 2 h. The tubes were centrifuged at 2000 rpm for 15 min. Supernatant (1 ml) was mixed with 0.075 ml 0.1 M EDTA solution and 0.25 ml of 1% thiobarbituric acid (TBA). All tubes were placed in a boiling water bath for a period of 15 min after which they were shifted to an ice bath. The amount of TBARS formed in each of the sample was assessed by measuring the optical density at 532 and 412 nm. The results were expressed as the nmol TBARS formed per gram of haemoglobin.

2.13.2. Tissue preparation

The isolated kidneys of rats were washed with ice-cold sodium chloride (0.9%). The tissues were homogenized in cold EDTA saline (2 mM) and the homogenate stored at −20 °C until further use.

2.13.2.1. Estimation of GSH. The GSH in kidney was determined by the method of Morgen et al. (1962). Homogenate (1 ml) was mixed with a 1 ml 10% TCA. The samples were incubated at 4 °C for 10 min and then subjected to centrifugation at 4000 rpm for 10 min at 4 °C. The assay mixture contained 1ml supernatant, 6 ml 0.3 M Na2HPO4 and 1 ml DTNB (0.238 mg/ml in phosphate buffer pH 8).

The yellow color developed was read immediately at 412 nm on a spectrophotometer. The GSH concentration was calculated as μg GSH/mg of protein. GSH of 100mg was placed in a 100-ml volumetric flask and brought to volume with reagent grade water. Calibrators of 50 and 10 mg/dl were prepared by diluting 5 ml of the 100 mg/dl calibrator with 5 and 45 ml, respectively, of reagent grade water. GSH concentration was estimated by using the following formula:

\[
\text{GSH (mg/dl of homogenate)} = \frac{\text{GSH concentration (calibration curve)}}{\text{hematocrit}}
\]

2.13.2.2. Assay for catalase activity. The catalase activity was measured by the method of Ellman (1959). The assay mixture consisted of 0.1 ml supernatant and 0.9 ml H2O2 (30 mM in phosphate buffer pH 7) in a final volume of 1 ml. Changes in absorbance were recorded at 240 nm and the catalase activity calculated in terms of μg H2O2/min/mg of protein.

2.13.2.3. Protein estimation. Protein concentration in all samples was determined by the method of Lowery et al. (1951).

3. Results

The LD50 of Viper russelli venom was established at 61 μg/mouse (20 g body weight) and the MND at 171 μg/rat (200 g body weight), respectively. Intraportal administration of the ethanol leaf extract of Acalypha indica did not produce toxic effects up to 5 g/kg in rats even after 24 and 72 h.

In vivo, studies the viper venom-induced lethality was antagonized by the ethanol leaf extract in a dose-dependent manner, with the dose of 750 mg/kg showing more significant protection than even snake venom antiserum (Table 1).

Venom-induced haemorrhagic and necrotizing activity was significantly inhibited by administration of the plant extract (Table 2). In cardiotoxic studies using the isolated frog heart, the ethanol leaf extract when added to the perfusate showed an initial fall in heart rate followed by an increase up to a dose level of 1600 μg/ml. Addition of viper venom (4 μg/ml) resulted in sustained cardiac arrest which was attenuated by the leaf extract from a dose of 400 μg/ml.

Table 1: Effect of the ethanol leaf extract of Acalypha indica on the lethality of Viper russelli venom

<table>
<thead>
<tr>
<th>Group (n = 8)</th>
<th>% Survival</th>
<th>% Increase in survival rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Snake venom</td>
<td>87.5</td>
<td>75</td>
</tr>
<tr>
<td>Ethanol leaf extract (250 mg/kg)</td>
<td>62.5</td>
<td>25</td>
</tr>
<tr>
<td>Ethanol leaf extract (500 mg/kg)</td>
<td>87.5</td>
<td>75</td>
</tr>
<tr>
<td>Ethanol leaf extract (750 mg/kg)</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of ethanol leaf extract of Acalypha indica on isolated frog heart in the presence of viper venom.

Table 2
Effect of the ethanol leaf extract of Acalypha indica on the Viper russelli venom-induced haemorrhage and necrotizing effects

<table>
<thead>
<tr>
<th>Group (n = 8)</th>
<th>MHD mean ± S.E.</th>
<th>MND mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.1275 ± 8.5E-03</td>
<td>5.1225 ± 1.1E-02</td>
</tr>
<tr>
<td>Ethanol leaf extract (500 mg/kg)</td>
<td>4.15 ± 6.5E-02</td>
<td>2.1100 ± 1.0E-02</td>
</tr>
<tr>
<td>Ethanol leaf extract (750 mg/kg)</td>
<td>3.1875 ± 6.57E-02</td>
<td>1.0950 ± 1.32E-02</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. control.

Table 3
Effect of the ethanol leaf extract of Acalypha indica on the Viper russelli venom-induced mast cell degranulation

<table>
<thead>
<tr>
<th>Group (n = 8)</th>
<th>Mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.5 ± 3.2E6</td>
</tr>
<tr>
<td>Snake venom antiserum</td>
<td>39.5 ± 0.6453</td>
</tr>
<tr>
<td>Ethanol leaf extract (500 mg/kg)</td>
<td>36.5 ± 0.6453</td>
</tr>
<tr>
<td>Ethanol leaf extract (750 mg/kg)</td>
<td>57.5 ± 1.04E9</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. control.

Table 4
Effect of ethanol leaf extract of Acalypha indica and snake venom antiserum on TBARS levels of blood in Viper russelli envenomed rats

<table>
<thead>
<tr>
<th>Group (n = 8)</th>
<th>TBARS (nm/gm of Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.27 ± 2.4E06</td>
</tr>
<tr>
<td>Snake venom antiserum</td>
<td>13.79 ± 6.1E-02</td>
</tr>
<tr>
<td>Ethanol leaf extract (500 mg/kg)</td>
<td>19.85 ± 2.3E-02</td>
</tr>
<tr>
<td>Ethanol leaf extract (750 mg/kg)</td>
<td>10.5200 ± 0.17E7</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. control.

In biochemical estimations of the blood, the extract at both doses significantly decreased the increased TBARS levels caused by viper envenomation (Table 4). Viper venom induced decrease in GSH and catalase levels of kidney tissue were significantly inhibited (Table 5).

Fig. 2. Effect of ethanol leaf extract of Acalypha indica on isolated frog rectus abdominus muscle in the presence of venom.

Cardiac arrest.

E1, E2, E3, E4 & E5 correspond to 100, 200, 400, 800 & 1600 µg/ml of ethanolic leaf extract of A. indica respectively.

V1, V2, & V3 correspond to 1, 2 & 4 µg/ml of viper venom respectively.

N- Normal heart rate
4. Discussion

Administration of the alcoholic extract of Acalypha indica at three dose levels, i.e. 250, 500 and 750 mg/kg body weight in mice, markedly reduced the mortality in mice. According to the WHO, the anti snake venom possessing compound should be tested regarding its capacity to neutralize venom effects such as lethality, haemorrhagic and necrotizing effects (Theakston and Reid, 1983). Most venom possesses the ability to cause local necrosis and haemorrhage when introduced intradermally. Hence, the minimum necrotizing dose and minimum haemorrhagic dose estimation proves a reasonable test for assessing the anti venom activity. Intravascular haemolysis may contribute to the development of acute tubular necrosis and bilateral cortical necrosis in victims of Russell’s viper bite. Haemorrhagins causes death because of bleeding from vital organs by damaging vascular endothelium (Warrell, 1989). The ethanol
extract of Acalypha indica was found to significantly reduce the viper venom induced necrotic and haemorrhagic lesions.

Neurotoxic action of vipersid venom causes respiratory failure and cardiac arrest with the dominant haemorrhagic action bringing about death (Khare et al., 1992). In our study, an attempt was made to determine the possible involvement of the cardiac system in the protective effect against Viper russelli venom. The alcoholic extract of Acalypha indica was found to prevent the cardiac arrest produced by venom up to a dose level of 400 μg/ml. Another toxic symptom of most snake venoms is the paralysis caused to the skeletal muscle. The results of the study on rectus abdominus muscle showed that the alcoholic extract of Acalypha indica did not modify either the normal muscle tension or the responsiveness of the muscle to acetylcholine.
Treatment with the alcoholic extract of *Acaclypha indica* was found to reverse the paralytic effect of *Viper russelli* venom in the skeletal muscle. Viper venom releases an enormous amount of histamine into circulation by mast cell degranulation. The released substances could also add to the various toxic signs and in fact may be responsible for some of the toxicity such as anaphylaxis. Mast cells are a rich source of mediators like histamine and platelet activating factors (PAF). These two mediators are implicated in several hypersensitive anaphylactic reactions and in the development of various toxic signs after snakebite (Slater et al., 1988). Phospholipase A2 present in viper venom might release a large amount of platelet activating factor in addition to histamine and other anaphylactic mediators from mast cells (Kannappa Reddy et al., 1993). In the present study, the protection of the mesenteric system against the degranulating effect of venom by the ethanol leaf extract may be by its interaction with phospholipase A2 and by prevention of the release of platelet activating factor and histamine from mast cells. It has been reported that antitoxin activity is one of the mechanisms of venom inactivation and inhibition (Alam et al., 1994; Alam and Gomes, 1998, 2003). In our study, blood TBARs levels were significantly reduced and non-protein sulfhydral (GSH) and catalase levels in kidney were significantly enhanced by the extract. Hence it may be postulated that the antivenom snake venom activity of this extract may at least be partly mediated through its anti oxidant property.

The crisis in snake venom and anti venom supply especially in the developing countries reflects a global loss of momentum in anti venom research, development and financing. In Maharashtra, use of prazosin (a pharmacological antitoxin to scorpion venom) as a cheap, simple, alternative with no risk of anaphylaxis has been successfully (Bawaskar and Bawaskar, 2001). Protection of animal rights in India mean that a new chemical antidote to snake envenoming such as prazosin should be sought. Our findings confirm the potent snake venom neutralizing capacity of the plant *Acaclypha indica*. Further study on isolation of active constituents and its anti snake venom activity could lead to the development of a new chemical antidote for snake envenoming.

**References**


