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Antivenom potential of ethanolic extract of *Cordia macleodii* bark against *Naja* venom

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PEER REVIEW

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Comments

This is a valuable research work in which authors have demonstrated the antivenom potential of ethanolic extract of *Cordia macleodii* bark against *Naja* venom. *Cordia macleodii* can be promising antidote against *Naja* venom poisoning.

Details on Page S453

ABSTRACT

Objective: To evaluate the antivenom potential of ethanolic extract of bark of *Cordia macleodii* against *Naja* venom induced pharmacological effects such as lethality, hemorrhagic lesion, necrotizing lesion, edema, cardiotoxicity and neurotoxicity.

Methods: Wistar strain rats were challenged with *Naja* venom and treated with the ethanolic extract of *Cordia macleodii* bark. The effectiveness of the extract to neutralize the lethalities of *Naja* venom was investigated as recommended by WHO.

Results: At the dose of 400 and 800 mg/kg ethanolic extract of *Cordia macleodii* bark significantly inhibited the *Naja* venom induced lethality, hemorrhagic lesion, necrotizing lesion and edema in rats. Ethanolic extract of *Cordia macleodii* bark was effective in neutralizing the coagulant and defibrinogenating activity of *Naja* venom. The cardiotoxic effects in isolated frog heart and neurotoxic activity studies on frog rectus abdominus muscle were also antagonized by ethanolic extract of *Cordia macleodii* bark.

Conclusions: It is concluded that the protective effect of extract of *Cordia macleodii* against *Naja* venom poisoning may be mediated by the cardiotoxic, proteolysin neutralization, anti-inflammatory, antiserotonic and antihistaminic activity. It is possible that the protective effect may also be due to precipitation of active venom constituents.

KEYWORDS

Naja venom, *Cordia macleodii*, Hemorrhagic activity, Necrotizing activity, Cardiotoxicity, Neurotoxicity

1. Introduction

Venomous snakebite is a WHO identified common acute medical emergency in most rural areas of the tropical and subtropical countries like India. Approximately 35000–50000 deaths are reported due to snakebite each year^[1]. India has rich assortment of snake fauna, of which 242 species have been identified including 57 poisonous or harmful species. The four major ubiquitous species of venomous snakes in India, known as “big four” are considered responsible

for life-threatening envenomation around the country. They include Indian cobra (*Naja naja*), the common krait (*Bungarus caeruleus*), the Russell’s viper (*Daboia russelii*) and the saw-scaled viper (*Echis carinatus*)^[2]. Many plants *viz.* *Mimosa pudica*^[3], *Mucuna pruriens*^[4,5], *Andrographis paniculata* and *Aristolochia indica*^[6–8], *Acalypha indica*^[9–12] are reported to inhibit krait and cobra venom activity. *Cordia macleodii* (Ehretiaceae), known as Dahiman in Hindi is a rare medicinal and timber plant found in moist, dry deciduous forests and successfully used for the treatment

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of snakebites by traditional healers in Chhattisgarh State of India. The leaves of the plant has been reported to have anti-inflammatory activity^[13]. The present study was aimed to examine the snake venom neutralization potential of ethanolic extract of *Cordia macleodii* bark to scientifically validate the traditional claim.

2. Materials and methods

2.1. Plant material and preparation of extract

The bark of *Cordia macleodii* was collected during the month of December 2009 from Marwahi Forest Division, Pendra Road, Bilaspur (Voucher number: SLT/Med. Plant/2009/15). The plant material was taxonomically identified and authenticated by Dr. Manoj Singh, Assistant Professor, Tulsi Mahavidyalaya, Anuppur (M.P). The shade dried coarsely powdered plant material (1.5 kg) was extracted with 70% ethanol in a Soxhlet apparatus. The extract was dried under vacuum (yield 8.7%). Preliminary phytochemical screening was carried out to detect the presence of steroids, carbohydrates, flavonoids, alkaloids, etc.

2.2. Experimental animals

Healthy adult male Wistar albino rats weighing about 180–250 g and Swiss albino mice weighing about 20–25 g between 2 and 3 months of age were used for the study. Animals were housed individually in polypropylene cages, maintained under standard conditions [12 h light and 12 h dark cycle; (25±3) °C]. The animals were fed with standard rat pellet diet (Pranav Agro Industries Ltd, Baroda, India) and water *ad libitum*. The study was approved by Institutional Animal Ethics Committee of the institute (Registration no. 994/ac/06/CPCSEA).

2.3. Venom

The *Naja* (*Naja naja*) venom was obtained from Kanan Pendari Zoo, Bilaspur, Chhattisgarh, India and was preserved at 4 °C. Before use, the venom was dissolved in saline, centrifuged at 2000 r/min for 10 min and the supernatant was used for antivenom studies. Venom concentration was expressed in term of dry weight.

2.4. Snake venom antiserum

Liquid snake antivenin (polyvalent) IP, Biological E. Ltd., Hyderabad (Batch no.: 90, Mfg Date: March 2009) was obtained from Primary Health Center, Pendra Road, Bilaspur.

2.5. Acute toxicity studies

The acute toxicity study was carried out as per OECD 423

guidelines. The limit test for acute toxicity was carried out at 2 g/kg oral dose. Nulliparous and non-pregnant female rats were used for the study and were observed continuously for 24 h for behavioral, neurological and autonomic profiles and, after a period of 24 and 72 h, for any lethality, morbidity state or death^[14].

2.6. Neutralization of lethal venom effect

The toxicity of *Naja* venom was assessed by *i.p.* administration of different concentrations of venom dissolved in 0.2 mL of physiological saline to groups ($n=6$) of Wistar albino rats (180–250 g). The median lethal dose (LD₅₀) of venom was determined 24 h later by the method of Theakston and Reid 1983^[15]. The antivenom potential of ethanolic extract of bark of *Cordia macleodii* (CME) was assessed by *i.p.* administration of LD₅₀ dose of venom into groups of rats ($n=6$), followed by *i.p.* administration of different doses of the plant extract. The standard reference group was administered snake venom antiserum after administration of LD₅₀ dose of venom.

2.7. Neutralization of hemorrhagic activity

The minimum haemorrhagic dose is defined as the least amount of venom which when injected intradermally into rats results in a haemorrhagic lesion of 10 mm diameter in 24 h^[15]. Neutralization of the haemorrhagic activity was estimated by mixing a fixed amount of venom with different amounts of CME. The CME-venom mixture was incubated at 37 °C for 1 h and 0.1 mL of the mixture was injected intradermally into mice. The haemorrhagic lesion was estimated after 24 h^[4].

2.8. Neutralization of necrotizing activity

The minimum necrotizing dose is the least amount of venom which when injected intradermally into rats results in a necrotizing lesion of 5 mm diameter in 3 d later^[15]. Neutralization of the necrotizing activity was estimated by mixing a fixed amount of venom with different amounts of CME. The CME-venom mixture was incubated at 37 °C for 1 h and 0.1 mL of the mixture was injected intradermally into mice. The necrotizing lesion was estimated after 3 d.

2.9. Neutralization of coagulant activity

The neutralization of coagulant activity was estimated by mixing different amount of CME, with a fixed amount of venom, incubating for 30 min at 37 °C^[4]. Different concentration of incubate were added to the experimental tube in place of 0.1 mL physiological saline, and the clotting time was recorded.

2.10. Minimum defibrinogenating dose (MDD)

The MDD of venom is defined as the minimum amount of venom injected *i.v.* into mice, causes incoagulable blood 1 h later^[15]. Neutralization of this activity was estimated as the amount of CME which prevented defibrinogenation by the MDD. Various amounts of CME were mixed with the MDD of the venom, incubated for 30 min at 37 °C and then injected *i.v.* into six mice (20–25 g). After 1 h mice were anesthetized with ether and bled by cardiac puncture. The coagulation was observed as described above. The neutralization ability of CME was expressed as the effective dose (ED), defined as the lowest CME/venom ratio in which blood coagulation occurred in the six mice.

2.11. Rat paw edema

The minimum edematous dose of venom/carrageenan is defined as the least amount of venom/carrageenan injected by intraplantar route to rats, produce inflammation in paw^[16]. Non fasted male albino rats (150–200 g) were treated with different doses of venom, carrageenan and CME. Venom (1 µg/kg)/carrageenan (1% in 0.01 mL) were injected in right hind paw after 15 min. As a control, equal amount of saline were injected in hind paw. The CME was administered orally. The edematous response was evaluated by the use of a screw gauge at given time intervals.

2.12. Neutralization of cardiotoxic activity

The effect of the CME on *Naja* venom induced changes in isolated frog heart was determined by the method of Kannappa *et al*^[17]. The isolated frog heart was connected to the perfusion apparatus containing Ringer solution, graded doses of the CME and *Naja* venom were injected and the changes in heart rate were recorded using kymograph (speed 12 mm/h, INCO instruments, Ambala, India). The effects of *Naja* venom in the presence of CME were also studied. The experiment was repeated in six isolated preparations^[9].

2.13. Neutralization of neurotoxic activity

The effect of CME on *Naja* venom induced changes on skeletal muscle was determined by the method reported by Kannappa *et al*^[17]. The rectus abdominus muscle of frog was mounted in an organ bath containing Ringer solution. The effect of acetylcholine, CME and *Naja* venom per se and the effect of acetylcholine and *Naja* venom in the presence of CME were recorded. The experiment was repeated in six isolated preparations.

2.14. Statistical analysis

Results are expressed as mean±SEM. The data were

analyzed by ANOVA complemented by the Tukey–Kramer test. Values of $P < 0.05$ were considered significant.

3. Results

The LD₅₀ of *Naja* venom was established at 120 µg/rat *i.p.*. The MHD was 240 µg/rat and the MND at 150 µg/rat respectively. Oral administration of the CME did not produce toxic effects up to 2 g/kg in rats after 72 h.

The data from *in vivo* studies indicate protection by CME against the *Naja* venom induced lethality in a dose-dependent manner. Significant protection was observed at 200 and 400 mg/kg. At the dose of 800 mg/kg CME showed higher protection than snake venom antiserum (Table 1). The rate of survived animals was increased to 100%.

Table 1

Effect of the CME on the lethality of *Naja* venom.

| Group (n=6) | % Survival | % Increase in survival rate |
|----------------------------|---------------------|-----------------------------|
| Control (Snake Venom) | 50.00 | — |
| Standard (Snake Antivenin) | 83.30 ^a | 66.60 |
| CME (200 mg/kg) | 66.00 ^b | 32.00 |
| CME (400 mg/kg) | 83.30 ^a | 66.60 |
| CME (800 mg/kg) | 100.00 ^a | 100.00 |

^a $P < 0.01$ vs. Control, ^b $P < 0.05$ vs. Control

The MND of venom injected intradermally into rat's shaved dorsal skin caused necrotizing and hemorrhagic lesions. The CME at 200 mg/kg reduced the necrotizing as well as hemorrhagic lesions significantly (Table 2). Blood treated with venom clotted faster than untreated blood. About 61.11 µg of *Naja* venom clotted human citrated plasma within 60 seconds. In the neutralization assay, the absence of clot formation shows the neutralizing ability of the CME. The CME at (1.81±0.11) mg of dose was able to completely neutralize coagulant activity (Table 3). The *Naja* venom also induced defibrination *in vivo* and this activity was neutralized successfully by CME at (2.10±0.21) mg dose (Table 4).

Table 2

Effect of the CME on hemorrhagic and necrotizing lesion.

| Group (n=6) | Hemorrhagic lesion | Necrotizing lesion |
|----------------------------|------------------------|------------------------|
| | (mm) | (mm) |
| Control (Snake venom) | 10.26±0.20 | 5.12±0.28 |
| Standard (Snake antivenin) | 0.00 ^a | 0.00 ^a |
| CME (200 mg/kg) | 6.18±0.08 ^b | 3.16±0.21 ^b |
| CME (400 mg/kg) | 4.78±0.20 ^a | 2.92±0.15 ^a |
| CME (800 mg/kg) | 3.25±0.21 ^a | 2.75±0.11 ^a |

^a $P < 0.01$ vs. Control, ^b $P < 0.05$ vs. Control

Table 3

Neutralization of coagulant activity.

| Venom | MCD (µg/mL) ^a | Effective dose of neutralization ^b (mg CME/mg venom) |
|-------------|--------------------------|---|
| <i>Naja</i> | 61.11±0.21 | 1.81±0.11 |

^aDefined as the concentration of venom (µg /ml) which induced coagulation of plasma in 60 seconds (mean±SEM; n=6).

^bDefined as the CME/venom ratio at which coagulation time was increased three times when compared to coagulation time of plasma incubated with venom alone (mean±SEM; n=6).

Table 4

Neutralization of defibrinogenating activity.

| Venom | MDD ($\mu\text{g/mL}$) ^a | Effective dose of neutralization (mg CME/ mg venom) ^b |
|-------------|---------------------------------------|---|
| <i>Naja</i> | 75.32 \pm 0.18 | 2.10 \pm 0.21 |

^aDefined as the minimum amount of venom which, when injected *i.v.* into mice, causes incoagulable blood 1 h later. Six mice were used per experimental group. ^bNeutralization ability of CME was expressed as effective dose, defined as the lowest CME /venom ratio in which blood coagulation occurred in the six mice.

Carrageenan and *Naja* venom induced significant edema in rat paw. Maximum inflammation was seen at 1 h which gradually decreased over a period of time. Intraplantar *Naja* venom injection followed by CME produced significant reduction in inflammation at 400 mg/kg dose (Table 5). The venom produced cardiotoxic effect and reduced the heart rate. The venom mixed with different amount of CME did not produce cardiotoxic effects. The effect was significant as compared to constant dose V3 of the venom. The protective action was increased with the increase in dose indicating the dose dependent protection (Table 6 and Figure 1).

Table 5Carrageenan and *Naja* venom induced edema and its inhibition by CME.

| Treatment | Edema (mL) | | | |
|-------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| | 1h | 2h | 3h | 4h |
| Carrageenan | 7.25 \pm 0.21 | 6.84 \pm 0.24 | 6.75 \pm 0.21 | 5.67 \pm 0.21 |
| <i>Naja</i> venom | 9.48 \pm 0.11 | 9.25 \pm 0.11 | 8.75 \pm 0.21 | 8.50 \pm 0.25 |
| Snake antivenin | 4.58 \pm 0.32 ^a | 4.67 \pm 0.27 ^a | 5.05 \pm 0.30 ^b | 5.30 \pm 0.20 ^b |
| CME (400 mg/kg) | 7.08 \pm 0.20 ^b | 8.25 \pm 0.11 | 8.25 \pm 0.11 | 8.25 \pm 0.21 |
| CME (800 mg/kg) | 5.08 \pm 0.15 ^a | 5.75 \pm 0.30 ^b | 7.25 \pm 0.17 | 7.50 \pm 0.12 |

^a $P < 0.01$ vs. *Naja* venom, ^b $P < 0.05$ vs. *Naja* venom

Table 6

Effect of CME on isolated frog heart.

| Dose | Heart rate/min |
|--------------------------------------|-------------------------------|
| Normal Saline (N) | 56.33 \pm 0.42 |
| C ₁ (100 μg) | 44.33 \pm 0.33 |
| C ₂ (200 μg) | 40.83 \pm 0.30 |
| C ₃ (400 μg) | 40.83 \pm 0.70 |
| C ₄ (800 μg) | 40.50 \pm 0.42 |
| C ₅ (1600 μg) | 42.60 \pm 0.84 |
| V ₁ (1 μg) | 34.32 \pm 0.51 |
| V ₂ (2 μg) | 28.22 \pm 0.49 |
| V ₃ (4 μg) | 21.13 \pm 0.42 |
| V ₃ + C ₁ | 24.11 \pm 0.21 |
| V ₃ + C ₂ | 26.41 \pm 0.32 |
| V ₃ + C ₃ | 34.50 \pm 0.50 ^b |
| V ₃ + C ₄ | 40.66 \pm 0.42 ^b |
| V ₃ + C ₅ | 48.50 \pm 0.76 ^a |

^a $P < 0.01$ vs. V₃, ^b $P < 0.05$ vs. V₃, C₁ to C₅ –Different doses of CME, V₁ to V₃: Different doses of *Naja* venom.

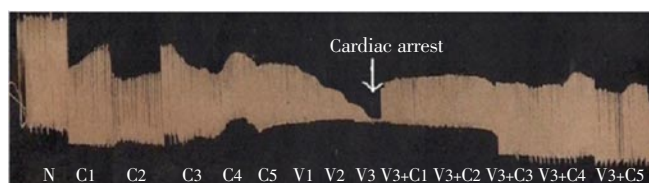


Figure 1. Effect of ethanol extract of *Cordia macleodii* on isolated frog heart in the presence of *Naja* venom.

In neurotoxic activity studies on frog rectus abdominus muscle, the addition of *Naja* venom (6 $\mu\text{g/mL}$) to the bath produced inhibition of the muscle to the stimulant effect of acetylcholine. This effect was significantly inhibited by the CME at the dose of 400 $\mu\text{g/mL}$.

4. Discussion

Snake bite remains a public health problem in many countries even though it is difficult to be precise about the actual number of cases[18]. Medicinal plants have been used for many years to treat a great variety of diseases including envenomations by animal bites. Therefore, experimental validation of the traditional use of plants is a necessary step in the development of low-cost phytotherapeutic agents. Many plants are recommended in Indian traditional medicine to treat snakebites some of which have been examined for neutralization of snake venom. The present investigation identified bark of *Cordia macleodii*, reported in Ayurvedic literature to have significant antivenom activity against *Naja* venom[6]. Administration of the alcoholic extract of *Cordia macleodii* bark at 200, 400 and 800 mg/kg body weight markedly reduced the mortality in rats. The *Naja* venom induced lethality was antagonized by CME in a dose dependent manner. Highly significant protection was observed at the dose of 800 mg/kg which was even more than the snake venom antiserum.

According to the WHO, the anti snake venom compounds should be tested regarding its capacity to neutralize venom effects such as lethality, hemorrhagic–necrotizing effects, neutralization of the coagulant and defibrinogenating activity[15]. Most venom possesses the ability to cause local necrosis and hemorrhage when introduced intradermally. Hence, the minimum necrotizing dose and minimum hemorrhagic dose estimation proves a reasonable test for assessing the antivenom activity[9]. Hemorrhagins of venom causes death because of bleeding from vital organs by damaging vascular endothelium[19]. The *Naja* venom contains most of the components which are responsible for the destruction of physiological process in the body. Several components are present in *Naja* venom like enzymes, proteolysins, cardiotoxins, hemorrhagins, cytotoxins and neurotoxins. The CME was found to significantly reduce the *Naja* venom induced necrotic and hemorrhagic lesions. In both the studies the CME showed highly significant results indicating the protective effect of CME against *Naja* venom induced lethality.

As a consequence of *Naja* bite local necrosis occur, which is often due to clot formation[20]. In patients who suffer from snake bites marked progress of defibrination has been observed[21]. CME effectively antagonized the clotting and defibrination induced by *Naja* venom indicating that CME possess protective effect against *Naja* venom poisoning.

The *Naja* venom containing one of the harmful components, proteolysin, is responsible for the local pain and swelling. In our study *Naja* venom induced the edema in rat paw indicating the edematogenic activity of the venom. The CME decreased the edema induced by venom indicating the antiedematogenic activity of CME. The *Cordia macleodii* has been reported to have antiinflammatory activity^[10]. The hesperetin-7-rhamnoside Lupa-20, isolated from *Cordia* species possess anti inflammatory and proteolysin neutralization activity^[22]. The CME might be showing antiedematogenic activity due to its anti inflammatory and proteolysin neutralization activity.

Naja venom contains large amount of cardiotoxin and phosphodiesterases. It is harmful for body, decreases heart rate and may cause cardiac arrest. In our study, an attempt was made to determine the possible involvement of the cardiac system in the protective effect against *Naja* venom. The CME was found to prevent the cardiac arrest produced by venom in dose dependent manner. The *Cordia* bark has been reported to have cardiotoxic activity and the aqueous extract was reported to produce significant positive inotropic and chronotropic effects on rat's heart^[23]. *Cordia* species has been reported to have cardiac glycoside, cordialin a, cordialin B. The preliminary phytochemical studies on CME indicated the presence of cardiac glycosides^[24]. The presence of cardiac glycosides might be responsible for the prevention of cardiac arrest and decrease in the heart rate.

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^[25]. The preliminary phytochemical analysis indicated the presence of flavonoids in CME. The flavonoids are reported to have anti-inflammatory, antihistaminic, antibradykinin and antiserotonin properties. The presence of flavonoids might be responsible for the protective effect shown by the CME against snake venom poisoning though it's anti-inflammatory, antihistaminic and antiserotonic activity^[26].

The *Cordia macleodii* bark is being successfully used for the treatment of snakebites in Chhattisgarh State. Extensive phytochemical studies carried out on this plant and wide class of compounds including flavonol glycosides, pyrrolizidine alkaloids, triterpenes, cardiaquinones, sphingolipids, cerebrosides were isolated^[19]. Hence it may be postulated that the anti-snake venom activity of CME might be mediated by the cardiotoxic, proteolysin neutralization, anti-inflammatory, antiserotonic and antihistaminic activity. It is possible that the protective effect against snake venom may also be due to precipitation of active venom constituents.

However more elaborate work is required to establish the efficacy of CME as potent anti-snake venom drug. Further experimental work is being carried out to isolate and identify the active principles present in the CME that are responsible for anti-snake venom activity.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Most of the snakebites recorded in India are caused by the *Naja naja*. Numerous studies are focusing on supplementary alternatives, such as the use of medicinal plants. *Cordia macleodii* has already demonstrated anti-inflammatory and healing properties. The present researchwork represents antivenom potential of ethanolic extract of *Cordia macleodii* bark against *Naja* venom.

Research frontiers

In the current study, the bark of *Cordia macleodii* neutralized the toxic activities of *Naja naja* snake venom and the activity was evaluated by using various parameters laid down by WHO.

Related reports

Snake venoms are a complex mixture of toxic enzymes and proteins such as phospholipases A, myotoxins, hemorrhagic metalloproteases, clotting serineproteases, neurotoxins, cytotoxins and others. Snakebite envenomations are frequently treated with parenteral administration of horse- or sheep-derived antivenoms aiming at the neutralization of toxins. Large number of plants have been reported to neutralize the snake venom toxicities.

Innovations and breakthroughs

Cordia macleodii is a well known medicinal plant used for the treatment of snakebite and cardiac tonic by traditional

healers. But no scientific data is reported for its use against snake bite. In present study, authors have scientifically tried to investigate the antivenom properties of *Cordia macleodii* extract against snake venom.

Applications

Despite the success of serum therapy, it is important to search for different venom inhibitors, either synthetic or natural, which would complement the action of antivenoms, particularly in relation to the neutralization of local tissue damage. Plant extracts constitute an extremely rich source of pharmacologically active compounds, and a number of extracts has been shown to act against snake venom.

Peer review

This is a valuable research work in which authors have demonstrated the antivenom potential of ethanolic extract of *Cordia macleodii* bark against *Naja* venom. *Cordia macleodii* can be promising antidote against *Naja* venom poisoning.

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