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Evaluation of Antivenom Activity of *Cassia alata* Leaf Extract against *Daboia Russelii* Venom

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Cassia alata, commonly known as candle bush, ringworm bush is an extensively distributed plant of the family *Leguminosea*. It is an annual and occasionally biannual herb, flowering in the sunshine and humid areas, with an average height of 1 to 4m. Biological activity in plants has been claimed to be effective as antidiabetic, antimicrobial, antioxidant, anti-inflammatory, anti-allergic, antimicrobial, analgesic, anti-ulcer, antiviral, antidepressants, hepatoprotective, antimalarial, anthelmintic, cardiovascular, and anesthetic properties. The ethanolic extract of plant was subjected first time for neutralization of snake venom activity. Primary phytochemical screening shows the existence of alkaloid, steroids, phenols, flavonoids, and triterpenoids. Ethanolic extract of *Cassia alata* plants was tested for antivenom activity against *Daboia russelii* venom. Various dose levels of leaf extract 200 and 400mg/kg showed significant neutralization of hemorrhagic activity, and at 400mg/kg leaf extract showed significant necrotizing activity in rats.

Keywords: Cassia alata; Daboia russelii; LD_{50}; venom; envenomation.

1. INTRODUCTION

There are 285 snake species found in Indian subcontinent, but only a few are venomous, and only four of them are accountable for the most of the fatal envenomations; Spectacled cobra (*Naja naja*), Common krait (*Bungarus caeruleus*),

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Russell's viper (*Daboia russelii*), Saw-scaled viper (*Echis carinatus*) [1].

The majority of medically important Indian snakes are these four species. India has around 2 subcategories of saw-scaled vipers, four subspecies of cobras, eight subspecies of kraits. All of which must be deemed relevant medically. To determine whether additional species of snakes in India need to be categorized as relevant, further studies are required. The grouping of several of these less known organisms is still not well known in order to complicate the problem. Various species of pit viper are known to cause severe morbidity and occasional mortality in local circumstantial accounts (mainly in northeastern India, where there are large numbers of pit vipers). There are two species of kraits, which are known to have deadly venoms, but to date, only the common krait (*Bungarus caeruleus)* venom is used for the manufacturing of antivenom [1].

Locally, the victim can experience a stinging sensation in the bitten area. This is quickly accompanied by pain, first of a dull, aching, and then of a type which lancinates and pierces. The final and quick outcome is the numbness due to the local paralysis. Slight swelling might also occur. Points or dots are commonly found in the case of cobra and Russel's viper, and other terrestrial reptiles, frequently seen by a thin film of clotted blood where the two fangs have penetrated half an inch or more apart. Or there would be only one point of entry, as in the case of a finger being bitten, the other fling having missed a later stage, the section assumes a lead or a livid shade, owing primarily to the effusion of blood under the skin (eccliymosis). The fang-marks are more difficult to discern whether the bite is caused by a salt-water snake; because the fangs are similar to fish teeth mark. Markings or scars of some of the teeth as well as that of the fangs could also be present. As the venom enters systemic circulation, the general symptoms disturbing the whole nervous system appears, resulting in confused focus and shadowed local signs. Failure to application of the ligature immediately following snake bite, systemic signs will appear quickly [2].

Snakebite envenomation is an important public health threat and the World Health Organization has announced snake bite as Neglected Tropical Disease in year 2017 [3].

Nearly 20% of snake bites are dry bites. The quantity of venom injected differs dramatically between species- Gaboon snake which delivers 450-600 mg of venom in each bite, is the highest in any snake. Snake venom is a complex material that can contain many toxins, depending on the species. Venomous snake bites can have various complications, that include pain, kidney failure, hypotension, respiratory depression tissue necrosis, swelling, blood clotting dysfunction, neuromuscular collapse, coma and can lead to death [4].

The only specific treatment for snake venom envenomation is antivenom immunotherapy. Antivenoms obtained from animals are hyper immune sera which will bind to venom components and render them inactive. Animal antiserum production is time-consuming, costly, and necessitates ideal storage condition [5].

Several efforts have been made over the years to develop remedies for snake bite, particularly from herbal sources. Plants have long been used to counteract the effects of snake bites; however, in the last 20 years, more scientific attention has been paid to this subject. Plants extracts have been used as a snakebite treatment by folklore healers, mainly in the tropical regions, where there are abundant sources of herbs available. Many herbs have been used as snakebite antidotes that appear in old drug recipes or have been passed down by oral tradition. Many attempts have been made in modern science to research these plants in order to determine their efficacy. The use of medicinal plants has a long history in India. One or more phytochemical in an herb may be accountable for antivenom potential. As a result, herbal drugs with antivenom activity can be a viable substitute to antivenom developed from animal source [5].

Snake venom is a multifaceted blend of enzymes, toxins, polynucleotide, non-toxic proteins, metals, lipids, carbohydrates, free amino-acids, nucleotides, and biogenic amines among others. Anti-snake venom (ASV) only neutralizes circulating venom; once venom is attached or absorbed to target organs, such as platelets, RBCs, renal tubules, vascular endothelium, muscles, and neuromuscular receptors, no amount of ASV can neutralize or combine with it. ASV is given early to avoid damage to the target organ. The excessive pouring of ASV will not prevent further degradation of vital functions in an effected organ [6].

Snakebite treatment varies from one species to the other. Using anti-venom against snake bites is the only widely accepted treatment. The first anti-venom (called an anti-ophidic serum) was discovered in 1895 against the Indian Cobra (*Naja naja*) by Albert Calmette, a French researcher at the Pasteur Institute. Anti-venom binds to the venom and neutralizes it, preventing further damage, but the damage already caused is not reversed. Any individual can develop early hypersensitivity reactions to the anti-venom. Other treatments for snake bite includes the use of conventional and folk remedies. Medicinal herbs are a regional heritage of global interest. In folk and herbal medicine, different plants have been used against snake bites. Distinctive plants and their compounds are stated to have anti-snake venom activity within the Ayurveda system. There are no systematic scientific study or literature available supporting the use of these herbs and folk medicinal plants [7].

Because of the high morbidity and mortality rates associated with snake bite, they are a major public health concern. Antivenom is the unique medication available. The search for complementary medication options for snakebite is therefore very relevant and appropriate. For several years, researchers have been searching for bioactive compounds extracted from the natural sources [8].

The most effective and acceptable treatment for snakebite patients is to administer antivenom following envenomation [9] and has been used for decades. Antivenom therapy, on the other hand has numerous adversarial effect on many organs of our body due to the injection of external proteins, presence of immune complex, and sensitization to horse serum [9,10].

Antivenom therapy carries two major risks;

- ‘Urticaria, gasping, hypotension, tachycardia, nausea and vomiting, diarrhea, angio-edema, upper airway edema, respiratory failure, shock and death are the symptoms of acute anaphylaxis that occurs when antivenom is administered within one hour [9,10].
- Serum sickness, which arises between 5-24days following antivenom therapy is a type III hypersensitivity response, which is caused by production of antigen- antibody complexes, usually causes fever, urticaria, itching, arthralgia, and usually treated with antihistamine, non- steroidal anti-inflammatory drugs and steroids. Neurotoxic symptoms may be seen in severe cases [10,11].

Furthermore, the conventional antivenoms have not always been able to resolve the local effects of the venom [9-12] such as haemorrhage, necrosis, local swelling, bacterial infections, pain, fever, and bleeding. Another issue with antivenom is its availability and high expense of treatment.

C.alata is also famous with its common name, for example, Emperor candlestick, candle bush. Also, the plant is known as Ewe Asunwon Oyinbo. Wild senna (*C.alata*) in Nigeria and is found in Ghana and Brazil, although it is now widely spread in the United States of America and throughout Africa, India [13,14].

It is a yearly and rarely biannual herb, flowering in the sunshine and humid areas, with a usual height of one to four meters. In Indonesia and Philippines, this plant is extensively distributed and grown for therapeutic uses [14].

Biological activity in plants has been claimed to be effective as antidiabetic, antimicrobial, antioxidant, anti-inflammatory, anti-allergic, antimicrobial, analgesic, anti-ulcer, antiviral, antidepressants, hepatoprotective, antimalarial, anthelmintic, cardiovascular, and anesthetic properties [15].

Certain chemical compounds like anthraquinone glycosides, flavonoids, phenolic compounds etc. are isolated from Cassia species. These chemical compounds are pharmacological markers, such as anti-inflammatory, hypolipidemic, hepatoprotective, antigenotoxic, purgative, hypotensive, anti-proliferative, anti-inflammatory, anti-diabetic, estrogenic, and anti-estrogenic, anti-diabetic, anti-ulcer, anti-oxidant, anti-helminthic, anti-mutagenic, antifungal, anti-bacterial, and anti-plasmodial [16].

The ethnobotanical survey revealed the importance of *C. alata* in the treatment of snakebite. But there is no scientific research is undertaken in this regard, hence this study has been carried out for the treatment of snake bite [16].

2. MATERIALS AND METHODS

2.1 Collection and Authentication of the Plant Materials

The leaves of *C.alata* were collected from the medicinal garden of the institute, during the

month of July 2020. The leaves were authenticated and voucher specimen was stored.

2.2 Preparation of the Extract

The collected leaves were washed and shade dried at room temperature. The dried leaves were subjected to maceration with ethanol.

The dried, grounded leaf was subjected to maceration by soaking in ethanol for 7 days and stirred occasionally for extraction. Ethanolic layer was filtered after 7 days. The complete extract's solvent was concentrated out, and the distillate was evaporated to a syrupy consistency on a water bath, and then evaporated to dryness, and stored on a desiccator until further use [17].

2.3 Selection of Animals

Healthy adult Wister albino rats, with body weight around 180-220g, and Swiss albino mice, with body weight around 20-30 g and aged between 8 to 12 weeks old were utilized for the present study. The study was presented to and permitted by the Institutional Animal Ethics Committee (NGSMIPS/IAEC/June-2020/197). The animals were grouped into six animals per cage. The animals were kept in a clean room maintaining a temperature of 25 ± 2°C and a dark and light cycle of 12 hours. The polypropylene cages covered by stainless steel grill were used. The paddy husks were used as a bed for the animal and the dirty cages were cleaned twice a week. The animals were fed with the regular pellet diet and water *ad-libitum.*

2.4 Pharmacological Investigation

2.4.1 Acute oral toxicity study

The acute oral toxicity was carried out in female Albino Rats (180 to 220g body weight) by Up and Down Method as per the OECD 425 guidelines. Before the experiment, animals were abstained from food overnight before extract treatment (water was provided but not the food). *C.alata* leaf extract suspended in sodium carboxymethyl cellulose(Na-CMC), and administered orally, at 2000mg/kg. The animals were then monitored for general behavioural changes for 3 hours, then every 30 minutes for the next 3 hours, and eventually death after 24 hours [18].

2.4.2 Selection of dose

Three dose levels were used to assess antivenom activity; a mid-dose that was 1/10th of the highest dose used in acute toxicity study, a low-dose, that was half of the 1/10th dose, and a high-dose that was double the 1/10th dose (200mg/kg,100mg/kg, 400mg/kg). For the antivenom study, plant extract was suspended in 0.6% Na CMC.

2.4.3 Preliminary qualitative phytochemical screening [19]

The test extract was subjected to the qualitative determination of phytoconstituents by using standard tests for alkaloids**,** Carbohydrate, Flavonoids, Glycosides, Triterpenoids, Saponins, Steroids**,** Phenols, and Proteins.

2.4.4 Evaluation of median lethal dose (LD_{50}) of the venom

The median lethal dose (LD_{50}) of *Daboia russelii* venom was assessed as per the previously described method [20].

The lethality of the venom was determined by intra peritoneal (i.p) injection of venom at various concentration, in 0.1 ml of saline. This venom was injected to groups (n=6) of albino mice. Mortality was observed after 24h of injection.

The LD_{50} was calculated using a probit analysis of deaths that occurred within 24 hours of venom administration, with a confidence limit of 50% [20].

2.4.5 Neutralization of Lethality

Neutralization of snake venom-induced lethality was studied as previously described [20]. Six groups of albino mice of either sex, weighing between 18-20g was selected for the study. Each group containing 6 mice. The animals were administered double the median lethal dose(MLD) of venom intraperitoneally (i.p), immediately the required dose of plant extract was given to the mice through oral route. All the animals were observed for mortality up to 24 hours. The number of surviving animals were recorded.

2.4.6 Inhibition of venom haemorrhagic activity

Minimum haemorrhagic dose (MHD) of *Daboia russelii* venom was assessed by previously described method in rats (21). The minimum haemorrhagic dose (MHD) of venom (defined as the minimum amount of venom that results in a

haemorrhagic lesion of 10 mm in diameter 24 hours later when injected intradermally into rat) was measured as per the previously described method. The neutralization of haemorrhagic action was measured by administering MHD of venom intradermally tailed by oral dosage of different doses of ethanolic leaf extract. The haemorrhagic lesion was measured after 24hr to determine the leaf extract's anti-haemorrhagic effect on venom.

2.4.7 Inhibition of necrotizing activity of venom

Neutralization of venom-induced necrosis was studied according to the mentioned procedure [21]. The minimum necrotizing dose (MND) (defined as the least amount of venom that results in a necrotic lesion of 5 mm diameter 3 days later when injected intradermally into rat) was determined as per the previously described method. Neutralization of the necrotizing activity was measured by administering MND of venom intradermally followed by ooral dosing of ethanolic leaf extract at various dose levels. The necrotic lesion was measured after 72hr to determine the leaf extract's anti-necrotic activity on venom [20].

2.5 Statistical Analysis

All the data were expressed as Mean ± SEM. The data obtained were subjected to a one-way Analysis of Variance (ANOVA) test followed using SPSS computer software version 16. P-values less than 0.05 were considered statistically significant.

3. RESULT

3.1 Preparation of Extract

The extract of the *C.alata* leaves was prepared as described earlier. A total of 300 grams of the dry coarse leaf powder was subjected to maceration using ethanol. The yield of *C.alata* leaf extract was found to be 32.07grms (10.69%).

3.2 Phytochemical Analysis of *C.alata* Leaf Extract

The extract was screened for the presence of Alkaloids, Flavanoids, Phenols, Glycosides, Steroids, Triterpenoid, Saponins, Tannins, Carbohydrates and Proteins by using standard chemical tests as mentioned earlier. The extract was tested positive for Alkaloids, Flavanoids, Triterpenoid, Steroids, and Phenols. The ethanolic leaf extract did not answer the tests for Carbohydrate, Glycosides, Saponins, and Proteins.

3.3 Pharmacological Investigation

3.3.1 Acute oral toxicity studies

The ethanolic extract of the *C. alata* leaf extract was found to be safe at the dose of 2000mg/kg body weight by oral route. The animals were observed for 24 hours for any sign of toxicity following oral administration of the extracts. Animals were found to be tolerated even after 24 hours. The leaf extract did not show mortality or any indications of toxicity and the extract was confirmed as safe.

3.3.2 Median lethal dose (LD_{50}) of the venom

The LD_{50} was found with the confidence limit at 50% probability by the analysis (probit analysis) of death's seen within 24 hours of the venom injection. The LD_{50} was found to be 20µg/20mg mice (i.p).

3.3.3 Neutralization of lethality

In-vivo venom neutralizing potency of *C.alata* plant extract was determined by i.p administration of 2 * LD_{50} of venom into various groups of mice, followed by oral administration of different doses of leaf extract. The leaf extract at doses 200 and 400mg/kg body weight were found to be effective in neutralizing lethality caused by $2LD_{50}$ of *Daboia russelii* venom (Table 1).

3.3.4 Neutralization of haemorrhagic activity

The MHD of *Daboia russelii* venom was determined to be 40µg, when injected intradermally in rats. The ethanolic extract of the leaf exhibited significant inhibition of haemorrhage at 200mg/kg and 400mg/kg dose levels (Table 2; Figs. 1 & 2).

3.3.5 Neutralization of necrotizing activity

The MND of venom was determined to be 50µg when injected intradermally. The leaf extract exhibited significant inhibition of necrosis caused by venom at dose level 400mg/kg (Table 3; Figs. 3 & 4).

Table 1. Effect of *C.alata* extract in mice administered with $2LD_{50}$ (40µg) of *Daboia russelii*

Group	Dose of the plant extract(mg/kg)	Mortality (after 24hrs)[no of death/no of mice used]	% survival after 24 hrs	Corrected %	Probit
1	Control(only venom)	6/6	-	4.16	3.25
3	100	3/6	50	50	5.00
4	200	3/6	50	50	5.00
5	400	2/6	66.66	66.66	5.44

Table 2. Effect of ethanolic extract of *C.alata* on *Daboia russelii* venom induced haemorrhagic activity in rat

Dose	Mean dia. Of lession±S.E
Control	10.833±0.401
100mg/kg	10.33±0.211
200mg/kg	6.500±0.342*
400mg/kg	3.167±0.167*

*The value is expressed as Mean ± SEM; n=6 rats in one group. *P< 0.05, when compared with control group.*

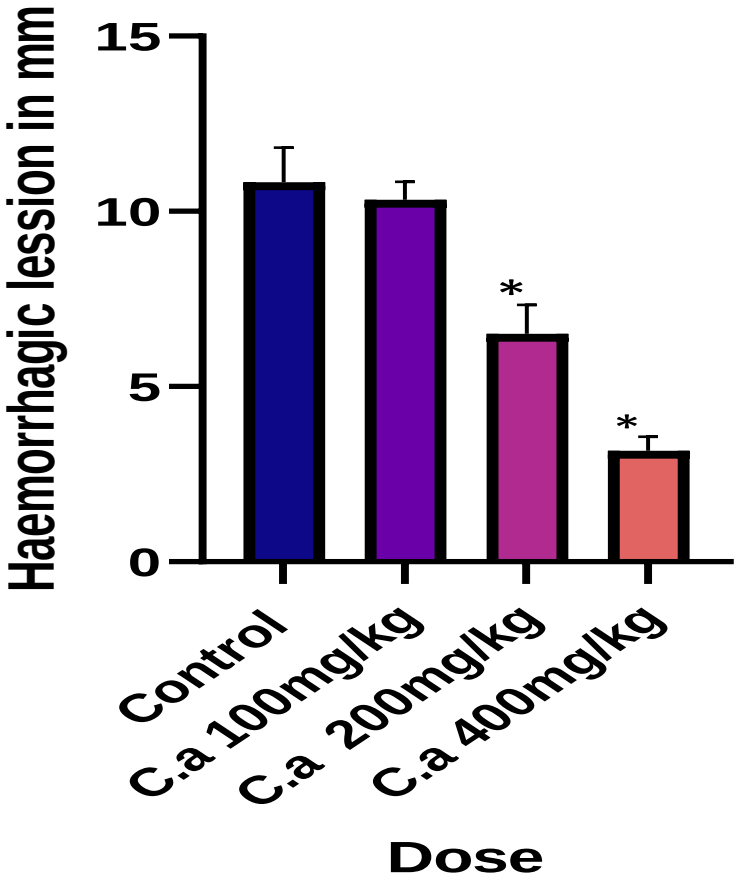

Fig. 1. Effects of *C.alata* extract on venom induced haemorrhage

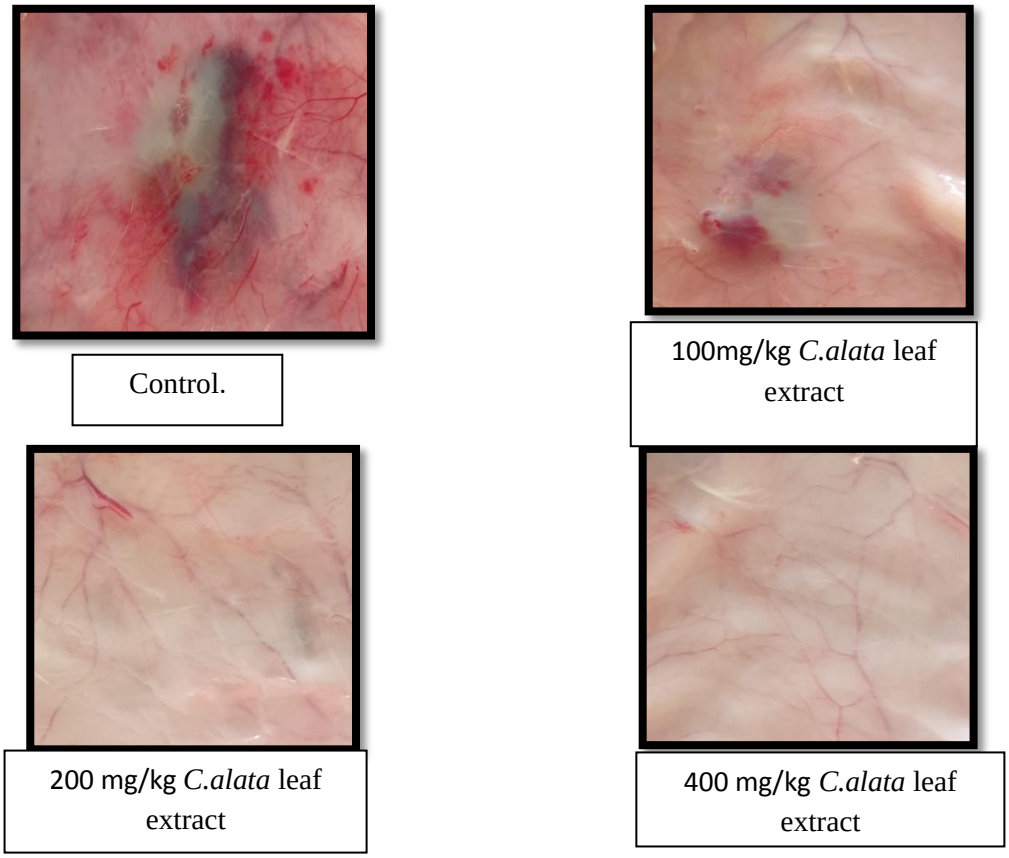

Fig. 2. Photographs showing venom induced haemorrhage and by extract of *C.alata* leaf.

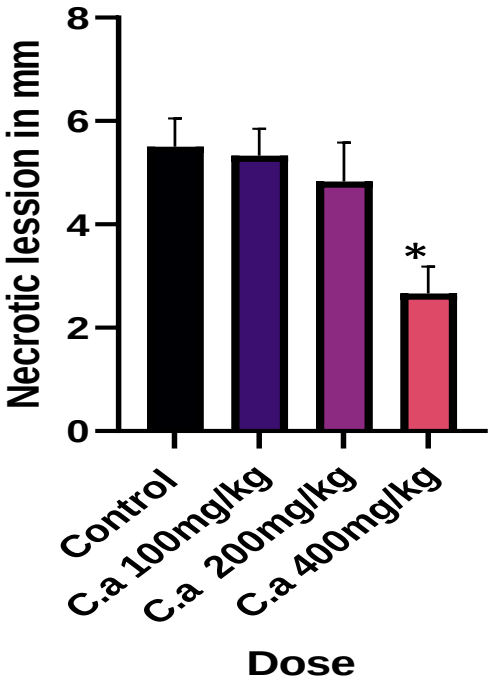

Fig. 3. Effect of *C.alata* extract on venom induced necrosis

Table 3. Effect of methanolic extract of *C.alata* on *Daboia russelii* venom induced necrotizing activity in rat

Dose	Mean dia. Of lession±S.E
Control	5.500±0.224
C.a 100mg/kg	5.333±0.211
C.a 200mg/kg	4.833±0.307
C.a 400mg/kg	2.667±0.211*

*The value is expressed as Mean ± SEM; n=6 rats in one group. *P< 0.05, when compared with control group.*

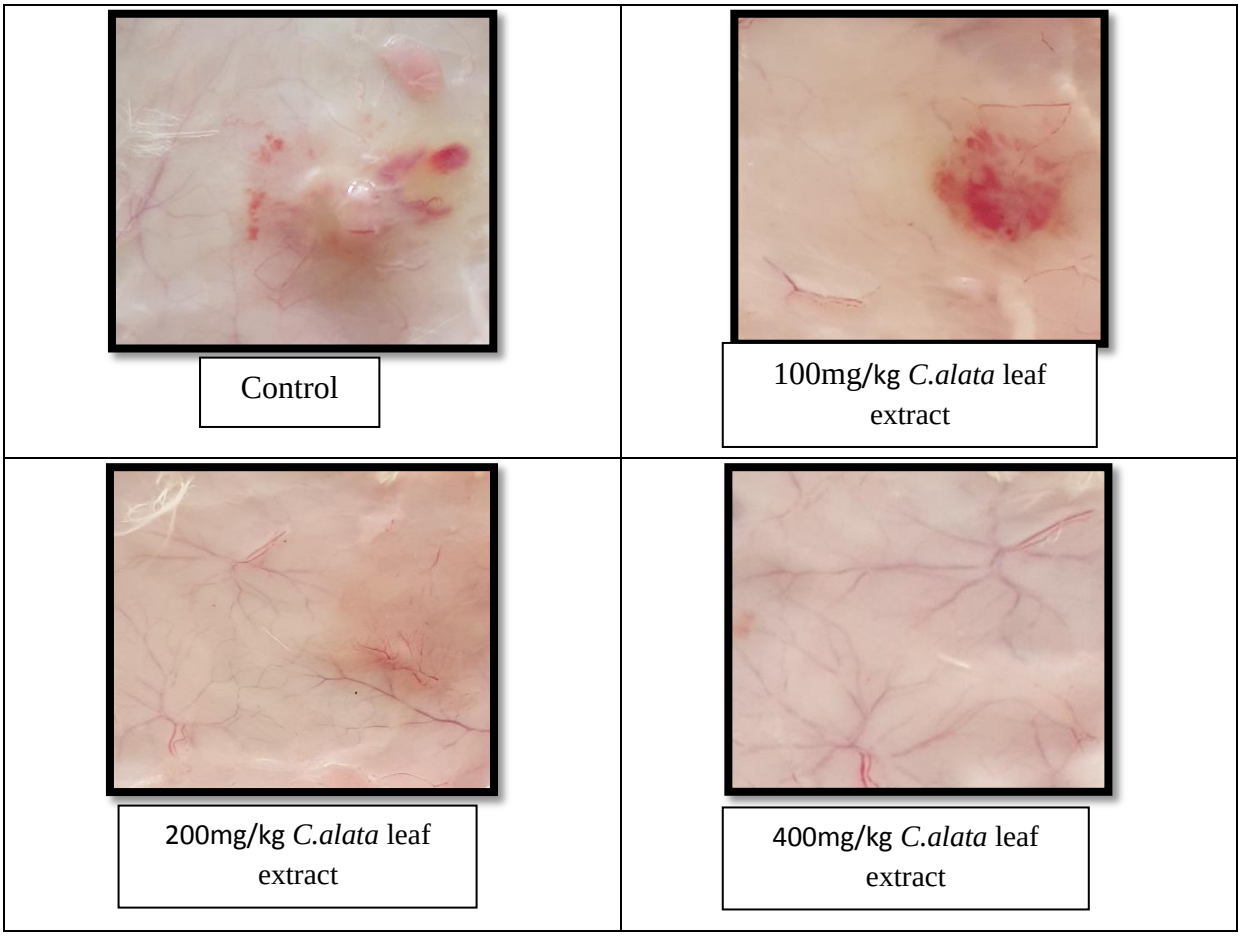

Fig. 4. Photographs showing venom induced necrotizing activity and its neutralization by the leaf extract

4. DISCUSSION

Around the world, snake bite is the one of the important Neglected Tropical Disease. Several venoms show high haemotoxic potential, and they interfere the blood pressure, clotting factors and platelets, and by directly causing haemorrhage and ultimately leading to death [22].

In folk and herbal medicine, different plants have been used against snake envenomation [9].

According to WHO, a compound that has the ability to neutralize the snake venom must be confirmed for its capability to inhibit the in-vivo toxic actions of the venom, such as necrosis, lethality, haemorrhage and edema [23].

In this study the ethanolic extract of *C. alata* leaf was used for assessing its ability to neutralize *Daboia russelii* venom. In the present study, based on acute toxicity studies in rats, three dosage of plant extract were chosen, ie., 100, 200, 400mg/kg body weight. LD_{50} of the venom

was found to be 20µg/20gm mice, when rats were injected intradermally, the MHD was 40µg after 24 hrs, and MND was found to be 50µg in rats after 3 days.

Ethanolic leaf extract when administered orally at dose 400mg/kg body weight, showed maximum protection against the lethality induced by $2LD_{50}$ of *Daboia russelii* venom. Significant inhibition of lethality was also observed at 200mg/kg of the leaf extract.

Daboia russelii venom when injected intradermally has the ability to induce local tissue necrosis and haemorrhage. Therefore, estimating minimum haemorrhagic and minimum necrotizing dose is a rational test for evaluating the venom neutralization ability. Intravascular haemolysis contributes for acute tubular necrosis and cortical necrosis in victims of *Daboia russelii* bite [23].

Nearly complete inhibition of haemorrhagic activity of *Daboia russelii* venom was observed at dose 200 and 400mg/kg of ethanolic extract of *C. alata* leaf. Similarly, complete inhibition of necrotizing activity was observed at dose of 400mg/kg of ethanolic extract of *C. alata*.

The global loss of momentum in antivenom research is seen in developing countries, it can also be due to lack of development and financing in the area of snake venom antivenom supply [24,25]. Furthermore, the conventional antivenoms have not always been able to resolve the local effects of the venom [11-14] such as haemorrhage, necrosis, local swelling, bacterial infections, pain, fever, bleeding, hence it reveals newer techniques to treat snakebite. The present study confirms that the ethanolic extract of leaf of *C. alata* have good snake venom neutralization capacity. Further research into the isolation and identification phytochemicals from the leaf extract, as well as its anti-snake venom activity could lead to development of new chemical antidote for snake bite.

5. CONCLUSION

In the present research work, antivenom activity of *C. alata* leaf extract was evaluated against the *Daboia russelii* venom. Form the results, we can conclude that, oral administration of ethanolic leaf extract of *C. alata* at 400mg/kg showed maximum protection of against the lethality induced by $2LD_{50}$ of *Daboia russelii* venom in mice. Complete inhibition of haemorrhagic activity was observed at dose 200 and 400mg/kg of ethanolic extract of *C. alata*. Similarly, complete inhibition of necrotizing activity was observed at dose of 400mg/kg of ethanolic extract of *C. alata*.

Our findings confirm good snake venom deactivation capability of ethanolic leaf extract of *C. alata,* therefore additional studies are needed to be carried out to evaluate the benefit of using *C. alata* which have been proved to have anivenom activity. Further the active constituents of this plant should be isolated and its anti-snake venom activity needs to be evaluated.

CONSENT

It is not applicable.

ETHICAL APPROVAL

IAEC approval was obtained before initiating the study (ref: NGSMIPS/IAEC/June-2020/197).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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