ORIGINAL ARTICLE

Comparative study of antitoxin activity of *Calotropis gigentea* Linn and *Cassia fistula* Linn against *Naja-naja* (cobra) venom

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Calotropis gigantea Linn and *Cassia fistula* Linn is used traditionally in India to treat snake bite patients. Hydroalcoholic extract of dried leaves of *Calotropis gigantea* Linn and *Cassia fistula* Linn was tested for haemolysis, procoagulant, oedema-forming activity against cobra (*Naja-Naja*) venom. The hydroalcoholic extract showed a significant inhibitory effect on haemolysis, procoagulant, oedema-forming activity. *Calotropis gigantea* Linn extract was significantly neutralize *Naja-naja* venom as compared to *Cassia fistula* Linn in a dose dependent manner. The present study suggests that hydroalcoholic extract of dried leaves of *Calotropis gigantea* Linn and *Cassia fistula* Linn possess compounds, which neutralize the activity of cobra (*Naja-Naja*) venoms.

Key words: Oedema forming, hemolysis, *Naja-naja,* procoagulant

INTRODUCTION

Snake bite causes major health problems that relates to high mortality rate especially in the rural areas of India, where basic health facilities are poor and as a result death are common. In India about 35,000 to 50,000 deaths occur every year due to snake bites.[1] Fifty-two poisonous species of snakes available in India, common poisonous snakes are Cobra (*Naja naja*), Krait (*Bangarus Caeruleus*), Russell's viper (*Daboia russelli*) and Saw Scaled Viper (*Echis Carinatus*).[2] The cobra venom are neurotoxin in nature, because it contains three proteins such as cardiotoxins, neurotoxins and phospholipase A2, which attack the central nervous system of patients and causes respiratory blockage, heart failure tissue damage and paralyzes the body. The most effective and accepted therapy for snakebite patient is immediate administration of specific polyvalent anti-venoms following envenomation. Generally, the anti-venoms do not provide enough protection against venom-induced local tissue damage and often associated with risk of anaphylaxis and serum reactions.[3] Most of these symptoms may be due to the action of high concentrations of non-immunoglobulin proteins present in commercially available hyper immune antivenom. From several years researches have made to developed snake venom antagonists using plant resources. Various Indian medicinal plants are recommended for use in for the treatment of snake bite and some of the plant extract have been reported against snake bite. Numerous plant species are used as folk medicine throughout the world to treat against snake bite.[4] Traditionally, *Calotropis gigantea* and *Cassia fistula* have been used against snake bite. Our present investigation explores *Calotropis gigantea* and *Cassia fistula* plant extract neutralises the effect of cobra venom.

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MATERIALS AND METHODS

Venom

The freeze-dried snake venom powder of cobra was obtained from Haffkine Institute, Mumbai and was stored at 4°C.

Animals and Experimental Setup

Wistar albino rats of either sex weighing between 100–200 gm were obtained from B.R.N.C.P. Mandsaur Animal house. The animals were stabilized for 1 week; they were maintained at standard condition at room temperature; 60±5% relative humidity and 12 hr light-dark cycle. They had been given standard pellet diet and water *ad libitum* - throughout the course of the study. The animal were handled gently to avoid giving them too much stress, which could result in an increased anti-inflammatory out put. The study was approved by the Institutional Animal Ethics committee.

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Collection of Plant

Leaves of *Calotropis gigantea* Linn and *Cassia fistula* Linn were collect from medicinal garden of B. R. Nahata college of pharmacy, Mandsaur in October 2010 and authenticated by prof. C. K. Ningwal Head of Department of Botany of P.G College, Mandsaur. Voucher specimen Number BRNCP/C/010/2010 and BRNCP/C/011/2010 has been deposited in the Department of Pharmacognosy.

Preparation of Extract

The extract was prepared by hot continuous percolation. The shade dried leaves of *C. gigantea* Linn and *C. fistula* were coarsely powdered, weighed and extracted with 50% ethanol and 50% water and after extraction it was evaporated on water bath. After drying, yield was calculated for each extract.[5,6]

Haemolysis Activity

The hyposaline-induced haemolysis was modified in the present study by venom-induced haemolysis. Blood was collected from healthy human volunteers by vein puncture and Ethylene di tetra amine (EDTA) was used as an anti-coagulant. The collected blood was washed three times with saline and 1% Human red blood cells (HRBC) was prepared. Lyophilized venom of *Naja-naja* was dissolved in physiological saline solution to make a stock solution of 100 µg/ml. Then 1 ml of venom (100 µg), 1ml of phosphate buffer (pH 7.2) and 1ml of 1% HRBC was taken in various tubes. To these tubes different concentrations of the various extracts of *Calotropis gigantea* Linn and *Cassia fistula* Linn leaves extracts (160, 340, 620 µg/ml) were added. The control samples were mixed with extract free solutions. The mixtures were incubated at 37°C for 30 mins and centrifuged at 1000 rpm for 3 mins. The absorbance of the supernatant was measured at 540 nm using spectrophotometer. The percent inhibition of haemolysis was calculated according to the equation:[7]

% Haemolysis:- {(A Control-A test)/A Control} × 100.

Procoagulant Activity

In the procoagulant activity 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 dose, 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 *Calotropis gigantea* Linn and *Cassia fistula* Linn leaves extracts. The mixtures were incubated for 30 min at 37°C. Then 0.1 ml of mixture was added to 0.3 ml of citrated plasma and the clotting times recorded. In control tubes, plasma was incubated with either venom alone or plant extracts alone. Neutralization was expressed as effective dose (ED), defined as the venom at which the clotting time increased three times when compared with clotting time of plasma incubated with two MCD of venom alone.[3]

Oedema-forming Activity

The minimum oedema-forming dose of *Naja-naja* venom was defined as the least amount of venom, which when injected subcutaneously into rats footpad results in 30% oedema within 6 hours of venom injection. The thickness of each footpad was measured every 30 min after venom injection with a low-pressure vernier caliper.[8] The ability of both plant extracts in neutralizing the oedema-forming activity were carried out by various dilutions of *Calotropis gigantea* Linn and *Cassia fistula* Linn plant extracts orally. Rats were injected subcutaneously in the right footpad with 50 µl of the mixtures, containing venom, where as the left footpad received 50 µl of PBS alone. Control rats were injected with venom in the right footpad and 50 µl of PBS in the left footpad. One hour after injection oedema was evaluated. It was expressed as the percentage increase in thickness of the right footpad compared to the right footpad of the control.[9]

Statistical Analysis

The data were expressed as mean±SEM. The data of edema forming activity were analysis of variance (ANOVA). *P* value less than 0.05 was considered as statistically significant.

RESULT

The antitoxin potential of *Calotropis gigantea* Linn and *Cassia fistula* Linn plant extracts were tested against cobra venom by using *in vivo* and *in vitro* methods. These extract at 160 µg/ml, 320 µg/ml and 640 µg/ml were evaluated for their anti venom activity through hemolysis. *Calotropis gigantea* Linn showed significant inhibition as compare to *Cassia fistula* Linn, it showed at 640 µg/ml 72.35% inhibition. This showed that cobra venom have phospholipase enzyme that has the ability to lyse human RBCs [Table 1].

The minimum coagulation dose was determined as the venom dose inducing clotting of plasma in 60 sec. About 40 µg of Cobra venom clotted human citrated plasma

Table 1: Haemolysis activity of extracts

Drug	Conc. (µg/ml)	Inhibition of haemolysis activity (%)
Control	Venom (100 µg/ml)	-
Calotropis gigantea Linn	160 µg/ml	34.77
	320 µg/ml	57.09
	640 µg/ml	72.35
Cassia fistula Linn	160 µg/ml	23.97
	320 µg/ml	43.84
	640 µg/ml	65.4

within 60 sec. In the neutralization assay, the absence of clot formation shows the neutralizing ability of both plant extracts. We found that 500 µg/ml *Calotropis gigantea* Linn and 1000 µg/ml *Cassia fistula* Linn of plant extracts were able to completely neutralise coagulant activity [Table 2].

In oedema-forming activity, rats immunized with cobra venom showed an increase in footpad thickness. About 7 µg of cobra venom induced edema formation within 3 h, which is considered as 100% activity. The oedema was reduced up to 30% when 750 mg of plant extracts per mg venom was given. No further reduction in the percentage of oedema was observed even when there was an increase in antivenom dose [Table 3].

DISCUSSION

Snakebite is a major health hazard that leads to high mortality rate especially in rural India. Anti-snake venom remains the specific antidote for snake venom poisoning. In India, Cobra *(Naja-naja)* snakes are commonly found that lead to higher mortality rates due to envenomation of cobra snakes. The only specific treatment of snake venom poisoning anti-snake venom (ASV) is used, which is usually derived from horse sera. They contain horse immunoglobulins, which frequently causes side effects, and other proteins that cause serum sickness and anaphylactic shock. Although use of plants against the effects of snakes bite has been long recognized, more scientific attention has been given since last 20 years. Many Indian medicinal plants are used for the treatment of snakebites.

The present investigation explored the comparative study of antitoxin activity of *Calotropis gigantean* Linn and *Cassia fistula* Linn against *Naja-naja* (cobra) venom.

Table 2: Procoagulant activity of extracts

Drug	Dose of venom and extract	Coagultion time (sec)
Control	40 µg/ml	58-60
Calotropis gigantea Linn	40 µg/ml+500 µg/ml	120
Cassia fistula Linn	40 µg/ml+1000 µg/ml	70-74

Various pharmacological activities like edema forming activity, hemolysis activity, procoagulant activity caused by cobra were carried out. Neutralization of these pharmacological effects was carried out using *Calotropis gigantea* Linn and *Cassia fistula* Linn plant extract. Neutralization studies can be performed by incubating of venom and plant extract prior to testing (pre-incubation method). Oral dose of plant extract neutralize the oedema-forming activity. Oral dose was chosen because that is the way people use these plants in traditional medicine.

Cobra venom contain phospholipase enzyme, which acts on membrane associated phospholipids liberated lysolecithin, which act on the membrane of human red blood cells and causes lysis of HRBC. The plant extract acts on phospholipase enzyme, which neutralizes the haemolysis activity.

Pro-coagulants cause blood coagulation to occur due to its thrombin-like effect and also it can cause the activation of Factor X to Factor Xa. The anti-coagulants prevent blood from clotting essentially due to the effect of the venom fibrinolysis or fibrinogenolysins or action of phospholipase on platelets or plasma phospholipids. About 40 µg of cobra venom clotted human citrated plasma within 60s. In the neutralization assay, the absence of clot formation was shows the neutralizing ability of both plant extracts. Plant extract act on Factor X to Factor Xa and prevent blood clotting caused by venom.

Cobra venom induces a prominent local edema cause in human and experimental animals, which is responsible for significant fluid loss. Injection of 7 µg cobra venom induced local skin ulceration, which caused loss of some edema fluid. Both the plant extracts neutralize the edema forming activity. *Calotropis gigantean* Linn gave the significant effect on 500 mg/kg, 750 mg/kg dose in comparison with *Cassia fistula* Linn gave at 750 mg/kg dose at 3rd hour, which showed 30% oedema inhibition equivalent to 100% oedema inhibition. This showed that *Calotropis gigantean* Linn is more potent.

In case of haemolysis activity of various doses of *Calotropis gigantea* Linn and *Cassia fistula* Linn. The *Calotropis gigantea* Linn gave the significant result in comparison

Table 3: Inhibition of edema in percentages

Time (hr)	Control	Extract of *C. fistula* 250 mg/kg (%)	Extract of *C. fistula* 500 mg/kg (%)	Extract of *C. fistula* 750 mg/kg (%)	Extract of *C. gigentea* 250 mg/kg (%)	Extract of *C. gigentea* 500 mg/kg (%)	Extract of *C. gigentea* 750 mg/kg (%)
0	0.66±0.03	0	0	0	0	0	0
1	1.33±0.03	0.2	1.57	6.24	3.45	4.12	10
2	1.63±0.04	4.26	5.5	14.27	14.2	8.1	18.3
3	1.83±0.03	9.05	15.4	29.08	17.23	23.62	38.18
4	1.86±0.02	14.30	22.3	35.5	24.10	30.3	55.3
5	2.06±0.02	22.59	32.26	46.78	35.5	41.94	59.68
6	2.36±0.02	35.91	42.96	58.45	47.19	53.52	69.01

with *Cassia fistula* Linn. The extracts of both plant showed effected Hemolysis activity in a dose dependent manner but *Calotropis gigantea* Linn is more potent at 160 µg/ml, 320 µg/ml, 640 µg/ml doses.

Comparison of *Calotropis gigantea* Linn and *Cassia fistula* Linn extracts showed procoagulant activity in a dose dependent manner and the *Calotropis gigantea* Linn gave the effected results on 500 µg/ml.

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