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Research Article

Isolation and Evaluation of Venom Neutralising Fraction from the Areal Parts of *Hedyotis corymbosa*

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ABSTRACT
Hediotys corymbosa is a well known plant used in the traditional medicine. Although it is a well established venom antidote in the folk medicine, its efficacy in this area is not scientifically evaluated. The areal parts of the plant were extracted using traditional method and fractionated using chromatography. The various fractions were then tested in vitro and the promising fractions were subjected to antivenom evaluation using mice. Freeze dried cobra venom (*Naja naja*) was employed for the study. Snake venom antiserum was used as the standard. Inhibition of coagulant activity, fibrinolytic activity and haemolytic activity were studied in vitro. In vivo evaluation was done for parameters like inhibition of lethality, myotoxicity, defibrinogenating activity, haemorrhagic activity and edema. The fraction showed very good neutralization of various venom activities in low concentrations.

Keywords: *Naja naja*, antivenom activity, *Hediotys corymbosa,* snake venom antiserum, myotoxicity, defibrinogenating activity, haemorrhagic activity.

INTRODUCTION

Deaths due to envenomation is a common problem in many parts of the world. Cobra, Viper and Krait are the important poisonous snakes found in India and Cobra bite has been reported to cause majority of such deaths[1]. A correct estimation of the number of snake bite victims is not possible because a considerable population depends on traditional medicine which is not accounted[2]. Venom antiserum or antivenin is the most accepted and popular method of treatment[3]. The therapy is costly and also bears the risk of untoward serum reactions including anaphylactic shock. Although India has a rich heritage of antidote plants, very less scientific validation has been done in this area. There have been reports that plants like *Mimosa pudica, Gloriosa superba, Vitex negundo, Boerrhavia diffusa* etc showed neutralising capacity against various snake venoms[4]. Many of such studies utilised the crude extracts for the evaluation and have not attempted to identify or isolate the fraction or compound responsible for the activity. Detailed evaluation of such folkloric plants will help in the development of safer yet effective alternatives to antiserum therapy[5]. In this context, *Hediotys corymbosa*, a popular antidote plant in folk medicine was selected for the scientific evaluation of anti venom activity.

MATERIALS AND METHODS

Venom: The freeze dried cobra venom was obtained from Irula snake catchers Industrial co-operative society, Chennai, India. It was dissolved in 0.9% saline and centrifuged. The supernatant was used as venom and expressed in terms of dry weight.

Plant extracts: The plant *Hediotys corymbosa* (HC) was collected locally from Kottayam, Kerala, India. For the preparation of plant extracts, method followed by the 'Visha vaidya's was adopted. The areal parts of the plant were shade dried, ground and extracted by boiling with water for 4 hours. Then it was filtered while hot through a muslin cloth and the filtrate was evaporated to get a dry residue. These dry extracts were then stored in a refrigerator until use.

Animals: Male albino mice (18-20g) were used for the experiments. All experiments were approved by the Institutional animal ethics committee (Approval no: 149/1999/CPSCEA).

Preliminary phytochemical analysis: The aqueous extract of HC was subjected to preliminary phytochemical screening to identify various constituents.

Fractionation of HC extract: The aqueous extract of HC was subjected to Thin Layer Chromatograhic analysis and the solvent system for column chromatography was optimised by trial and error method. 5g of the extract was then loaded to a column (1.5 m length & 30 mm diameter) packed with silica gel and allowed to run until clearly separated bands were developed. Various fractions thus obtained were dried and used for the activity studies.

In vitro evaluation of antivenom activity

Neutralisation of coagulant activity[6]: The minimum

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Table.1. Phytochemical screening of HC extract

Phytoconstituents	HC
Alkaloids	+
Glycosides	–
Tannins	+
Flavonoids	+
Carbohydrates	+
Proteins	+
Steroids	+
Saponins	+

Table.2. Effect of HC fractions on venom induced coagulation

Dose (mg)	Coagulation time in seconds* shown by fractions		
	H.C-1	H.C-2	H.C-3
1	68 ± 1.4	53 ± 2.4	54 ± 1.8
2	89 ± 2.5	59 ± 1.6	63 ± 3.0
3	108 ± 1.8	65 ± 1.9	69 ± 2.2
4	130 ± 3.0	73 ± 2.6	76 ± 1.8
5	145 ± 2.2	78 ± 2.4	84 ± 2.8

**Average of 3 independent observations*

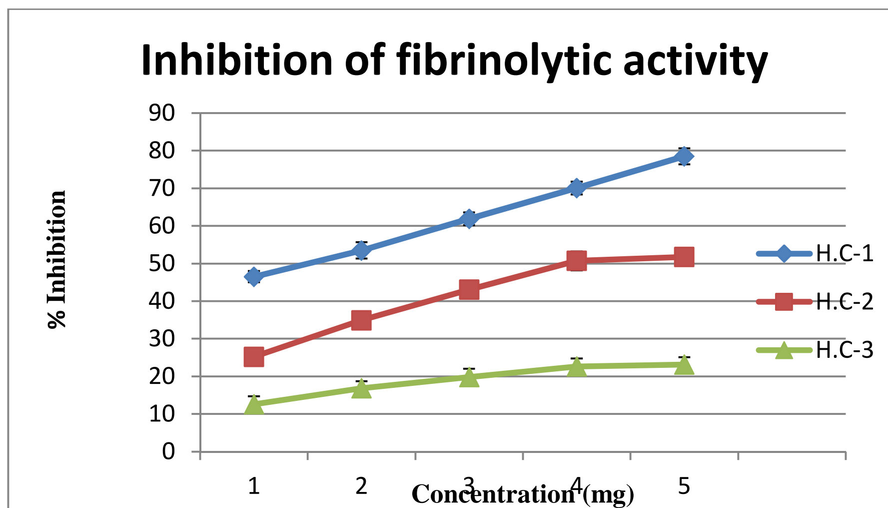

Fig. 1: Inhibition pattern of various HC fractions against cobra venom induced fibrinolysis

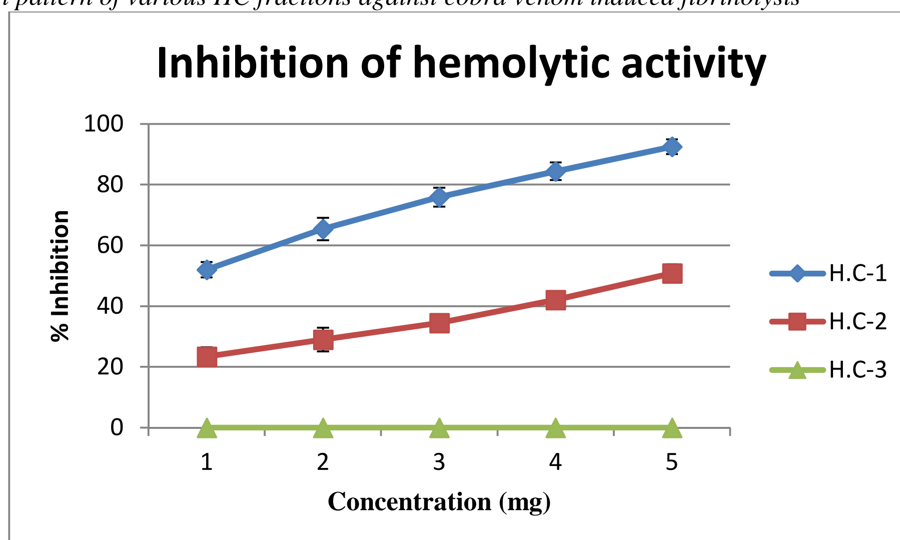

Fig. 2: Pattern of inhibition of venom induced hemolysis by various HC fractions

clotting dose of plasma (MCD) was determined by the modified method of Laing et al., 1992. Various amounts of venom dissolved in 100µl PBS (pH 7.2) was added to human citrated plasma at 37^{0}C. Minimum coagulant dose was determined which is the concentration of venom that induced clotting of plasma within 60 seconds. For the neutralization experiments, various doses of fractions were incubated with 2MCD of venom for 30 minutes at 37^{0}C. A measured volume from the incubate was then added to citrated plasma and the clotting times were recorded. Plasma incubated with venom alone served as the control.

Inhibition of fibrinolytic activity[7]: Fresh blood of sheep was collected and coagulated in a petridish, dried and cut in to various pieces of 100 mg. Each were then transferred to separate amber bottles containing venom and venom-extract mixture in 2 ml of PBS, incubated for at 37^{0}C. After 24 hours, the clots were taken out, dried and re-weighed. The percentage inhibition of clot dissolution by various doses of fractions was calculated in comparison with control and the fractions which showed maximum inhibition were selected.

Inhibition of haemolytic activity[8]:For the estimation of in vitro haemolytic activity, spectrophotometric method was used. 0.5 ml of a 0.5 % erythrocyte suspension was mixed with 0.5 ml of a mixture of venom with various concentrations of fractions in PBS. The mixture was then incubated at 37^{0}C for 30 min and centrifuged at 1500 rpm. The free haemoglobin in the supernatant was measured at 540 nm. Erythrocyte suspension treated with venom alone was used as the control. % inhibition of haemolysis was calculated and the fractions which showed maximum inhibition of hemolysis were selected.

In vivo evaluation of antivenom activity

Inhibition of edema[9]: The minimum edema forming dose was the dose of venom that induced 30% edema within 6 h. Various groups of mice were given sub plantar injection to the right foot pad with venom-fraction mixture (fixed dose of venom preincubated with various doses of fractions at 37^{0}C for 60 min) dissolved in 50 µL phosphate buffer saline. A group of mice receiving venom alone served as the positive control while the left foot pad injected with 50 µL phosphate buffer saline was kept as normal control. A group treated with Diclofenac sodium was used as the standard. Edema was calculated as the percentage increase in the volume of right foot pad when compared to left foot pad.

Inhibition of defibrinogenating activity[10]: MDD is defined as the minimum amount of venom that causes 1 h later, incoagulable blood when injected i.v in to mice. To determine MHD, various concentrations of venom were administered i.v to different groups of mice and the coagulation was observed. The neutralisation experiments were performed using a fixed amount of venom and various concentrations of fractions preincubated at 37^{0}C for 60 min.

Inhibition of myotoxic activity[11]: Myotoxic activity is estimated in terms of plasma creatinine phospho kinase. The fractions were mixed with a fixed dose of venom and incubated at 37^{0}C for 60 min. Aliquots of this mixture was then injected in to the right gastroenemius of mice and after 4 h, blood samples collected from the tail vein were used for the measurement of plasma CPK activity. Enzymatic activity exhibited by venom alone was taken as control and venom antiserum was used as the standard.

Inhibition of haemorrhagic activity[12]: The minimum haemorrhagic dose (MHD) is defined as the least amount of venom which results in a haemorrhagic lesion of 10 mm diameter, 24 h later when injected intradermally in to mice. To determine MHD, various concentrations of venom were administered intradermally to different groups of mice and the diameters of haemorrhagic lesions were measured.

Inhibition of lethality[13]: Lethal dose of venom was determined by intra peritoneally injecting various concentrations of venom dissolved in physiological saline in to different groups of albino mice. For the inhibitory studies, various fractions were preincubated with a fixed dose of venom at 37^{0}C for 60 min followed by injection in to mice and percentage survival in various groups was determined. Snake venom antiserum was administered i.v to the standard group.

Statistical analysis: All results are expressed as mean ± SD and ANOVA is applied to interpret the results wherever applicable.

RESULTS & DISCUSSION

Preliminary phytochemical screening: Qualitative analysis of the aqueous extract of HC revealed the presence of alkaloids, tannins, carbohydrates, flavanoids, steroids, proteins and saponins.

Chromatographic analysis: Thin layer chromatographic analysis was conducted on HC extract to optimise the solvent system for column chromatography. The solvent system chloroform: Methanol (6:4) was found to be ideal as it gave best resolution. Further development of HC extract in a silica gel column using the optimised solvent system resulted in three fractions namely H.C-1, H.C-2 and H.C-3.

In vitro evaluation

Coagulant activity: The minimum coagulant dose of cobra venom was 60 µg. Venom caused clotting in the positive control tubes at 45 ± 2 sec while in the normal tubes, clotting was effected at 155 ± 3.5 sec. All the fractions exhibited dose related response on the restoration of coagulant activity. However, H.C-1 was selected as the best as it brought back the coagulation time to almost normal with an average of 145 ± 2.2 sec.

Fibrinolytic activity: Venom acts in the various stages of coagulation and also exhibits prominant action on the fibrin clot formed. The experiment is based on the fact that venom causes the breakdown of fibrin in vitro. 10 µg of cobra venom was used for the study, the activity of which is considered as 100% fibrinolysis. The three fractions of H.C showed varying degrees of inhibition. H.C-1 showed maximum inhibition with 78.5% while H.C-3 showed least activity with inhibition as low as 23.1%.

Inhibition of hemolytic activity: Minimum hemolytic dose was found to be 15 µg. H.C-1 showed excellent inhibition of hemolysis (92.5%) while H.C-2 showed only moderate activity (50.8%). H.C-3 was totally ineffective in neutralising the venom induced hemolysis.

Table 3. Effect of HC fractions in venom induced mouse paw edema

Groups	Paw volume at 1 hour	2 hour	3 hour	4 hour
Normal	1.13 ± 0.042	1.14 ± 0.067	1.13 ±0.077	1.13 ± 0.056
Positive control	1.17 ± 0.061	1.64 ±0.075	1.84 ±0.086	1.72 ± 0.067
H.C- 1	1.13 ± 0.084	***1.34 ±0.081	***1.27 ±0.074	***1.15 ± 0.083
H.C-2	1.14 ± 0.072	1.57 ±0.067	1.50 ±0.049	1.35 ± 0.037
Standard	1.15 ± 0.045	***1.46 ±0.048	***1.29 ±0.077	***1.18 ± 0.055

Statistical significance calculated w.r.t Positive control

Table 4: Inhibition of venom induced defibrinogenation by HC fractions

Groups	Coagulation pattern of blood	Coagulation time (s)
Normal	Normal	148 ± 2.0
Control	Not coagulated	>300
H.C-1	Normal	138 ± 1.7
H.C-2	Not coagulated	>300

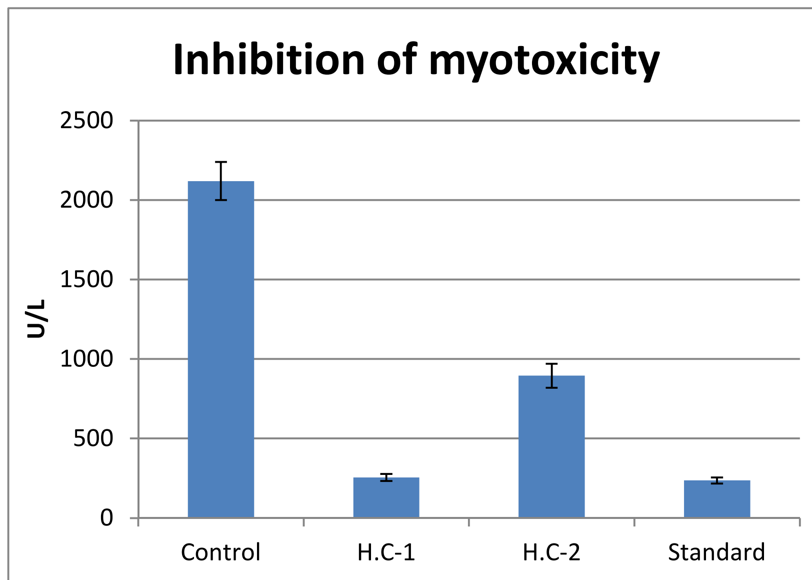

Fig. 3. Graph showing CPK enzyme inhibition by various HC fractions

Groups	Treatment given	No. of mice survived at 24 h
Normal	Normal saline (i.p)	6/6
Control	Cobra venom (i.p)	0/6
H.C-1	Incubate of Cobra venom + H.C-1 (i.p)	6/6
H.C-2	Incubate of Cobra venom + H.C-2 (i.p)	2/6
Standard	Cobra venom (i.p) + Antivenin (i.v)	6/6

In vivo activity: Among the three fractions of H.C, H.C-1 and H.C-2 were selected for in vivo studies based on the results of in vitro evaluation. In all the experiments, six mice were used per group.

Inhibition of edema: 3 µg of cobra venom was identified as the minimum edema forming dose. All the groups except the normal group showed a sharp increase in paw volume at the 2nd hour and varying degrees of edema reduction by the 3rd and 4th hour. H.C-1 presented excellent activity, equal to standard while the activity of H.C-2 was not significant.

Inhibition of defibrinogenating activity: Minimum defibrinogenating dose of cobra venom was found to be 5 µg. Administration of MDD of cobra venom in control animals resulted in incoagulable blood after 1 h. In group treated with H.C-1 and standard, bleeding stopped at 138 ± 1.7 s and 141 ± 2.2 respectively. In the control and H.C-2 groups, bleeding continued for longer time and normal coagulation of blood was not achieved.

Inhibition of myotoxic activity: Myotoxicity of venom is due to the action of Creatinine Phospho Kinase enzyme. H.C-1 showed significant neutralisation of these enzymes and the result was comparable to the standard. The activity of H.C-2 was not significant.

Hemorrhagic activity: Cobra venom failed to produce hemorrhagic spot in mice up to 8 µg. Higher doses of venom resulted in death.

Inhibition of lethality: The lethal dose of venom was found to be 8 µg/20g of mouse. 16 µg was used for the study. H.C-1 and H.C-2 (10 mg/Kg b.w) was mixed with cobra venom in physiological saline and incubated at 37^0C for 30 min. All animals in the positive control group died within

24 h while all animals in the H.C-1 and standard survived. H.C-2 could not produce 100% survival in the group.

DISCUSSION

H. corymbosa is a common field plant found throughout the tropical and subtropical parts of India and is used by the visha vaidyas for treating the snake bite patients. The present study underlines the utility of aqueous extract of HC in neutralising various toxic effects and lethality of *Naja naja* venom. It is long established that the lethal toxicity of venom is due to its enzymes which cause local pathological effects like myonecrosis, muscular degeneration etc[14]. Therefore, antivenom compounds should show complete or partial neutralisation of these venom effects. Although, ASV (anti snake venom or venom antiserum) is popular and effective, an alternative is much anticipated due to its limitations. Many herbs have been scientifically proved to be venom antidotes. The advantages of herbal compounds are that they are safe, cheap, stable and do not require critical storage conditions. They are also capable of potentiating the action of ASV. Thus, herbal antidotes may help in the snake bite therapy either alone or as an adjuvant to ASV.

Present study was aimed at isolating and identifying the fraction which has potential for venom neutralisation. The isolated fractions were tested against all important properties of venom such as hemotoxicity, myotoxicity, edema and lethality and the best fraction in terms of *in vitro* and *in vivo* venom neutralisation was identified. The H.C-1 fraction showed promising results by achieving 100% inhibition of lethality in experimental animals. However, it is noteworthy that the venom- fraction reaction was in vitro and so the same success may not be obtained if venom and test fraction are administered through different routes without pre incubation.

Some herbalists advocate and support the use of whole herbal extracts instead of isolated compounds or fractions. They believe that the whole extracts are more powerful due to the probability of synergism and antagonism of various constituents present in the plant. On the other hand, modern medicine employs and supports the use of isolated compounds and spreads the concept that isolated compounds are superior to crude extracts[15]. In the present study, isolated fractions were used for activity studies with an intention to identify the entity responsible for the anti venom activity. Further research may be done on the whole extract of this plant to compare and establish its inferiority or superiority with respect to various fractions. Future research should also encompass the characterisation of these fractions with the help of modern chromatographic and spectroscopic techniques.

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