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Original Research Article

In Vitro Neutralisation Effect of Acorus Calamus and Hibiscus Rosa-Sinensis Extracts on Viper Venom

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ABSTRACT

Snakebites represent a public health hazard that leads to high morbidity and mortality in the Indian subcontinent. The common poisonous snakes found in India are Cobra (*Naja naja*), Krait (*Bungarus Caeruleus*), Russell's viper (*Daboia russelli*) and Saw Scaled Viper (*Echis Carinatus*). Antivenom immunotherapy is the only specific treatment against snake venom envenomation. The use of plants against the effects of snakes bite has been long recognized; more scientific attention has been given since last 20 years. Methanolic extracts of *Acorus calamus* and Ethanolic extracts of *Hibiscus rosa-sinensis* were tested for their activity on pharmacological effects like Caseinolytic activity and Procoagulation activity exhibited by the Viperid venoms. *Hibiscus rosa-sinensis* extracts showed a significant inhibitory effect in both assays whereas *Acorus calamus* showed a significant effect only in the Caseinolytic assay. The present finding suggests that extracts of *Hibiscus rosa-sinensis (leaves)* and *Acorus calamus (roots)* possess compounds which inhibit the effect of the enzymatic components present in the venom of the Viperidae family. Further investigations are needed for identification and purification of the active components involved in the neutralization of the viper venom.

Keyword: Pro coagulant assay, Caseinolytic assay, venom, Russell's viper.

INTRODUCTION

Snakebites represent a public health hazard that leads to high morbidity and mortality in the Indian subcontinent. In India alone more than 200,000 cases are reported and an estimated 35,000 to 50,000 people die each year. [1,3,4] The common poisonous snakes found in India are Cobra (*Naja naja*), Krait (*Bungarus Caeruleus*), Russell's viper (*Daboia russelli*) and Saw Scaled Viper (*Echis Carinatus*). The only method to combat this problem is to produce effective antivenin. [2,5] Antivenin is an antitoxin active against the toxin of a snake, spider or any other venomous animal. After milking the venom from the desired animal, it is diluted and then injected into large animals such as cows or horses. The tiny amount of venom doesn't harm the larger animals but instead leads to the rapid production of antibodies. These antibodies then tag the toxins for destruction by other parts of the immune system. After a few weeks, blood from the injected animal is withdrawn and serum is isolated from it. This serum is rich in antibodies. The antibodies are purified and then administered to patients. There are various side effects of antivenin such as anaphylactic shock, pyrogen reaction and

serum sickness. Most of these symptoms may be due to the action of high concentrations of non-immunoglobulin proteins present in commercially available hyper immune antivenin. Over the years many attempts have been made for the development of snake venom antagonists especially from plants sources. [6,7] Medicinal plants represent an important source of bioactive compounds able to help directly in the treatment of ophidian envenomation, or indirectly, [8,9] as supplements to conventional serum therapy. Many Indian medicinal plants have been named in the ancient folklore for the treatment of snakebite. [10] This project aims at finding the venom neutralization activity of *Hibiscus rosa-sinensis* and *Acorus calamus* against the venom of the Russell's viper by in vitro methods.

MATERIALS AND METHODS

Materials

Snake Venom

Freeze dried Russell's viper venom was obtained from Haffkine Institute, Parel, Mumbai and was preserved at -20^{0}C for further use.

Figure 1: Snake venom Extraction

Plant Material

The leaves of *Hibiscus rosa-sinensis* were collected from the Haffkines Institute campus (Parel), whereas the rhizome of *Acorus calamus* was bought from a vendor specializing in herbs and herbal products at Vashi. Both, the leaves and the rhizomes were shade-dried and powdered in a mixer grinder. [12-15]

Methods

Preparation of plant extracts

The plant material was shade dried for 7 to 8 days and then powdered in a mixer grinder. The cold extracts were prepared using Methanol and Ethanol as the extraction solvent for Acorus calamus and Hibiscus rosa-sinensis respectively. The cold extracts were kept on rotary shaker at room temperature. Next day the solvent was first filtered with filter paper, then with a muslin cloth and finally with a Whatman Paper (grade 1). The recovered solvent was transferred into a beaker and then kept for evaporation in a water bath set at a temperature of 40^{o}C. The crude extract thus obtained was then used for further analysis. [16,17]

Figure 2: Leaves of Hibiscus rosa-sinensis

Preliminary phytochemical screening

The methanolic cold extracts obtained were subjected to phytochemical screening for its constituents by standard methods as described by Saiprasanna *et. al.* 2012. [18-21]

Figure 3: Rhizome of *Acorus calamus*

Assessment of the phytochemical constituents of the extracts using HPTLC

The phytochemical constituents of Methanolic cold extracts were also subjected to High Performance Thin Layer Chromatography (HPTLC) screening on HPTLC pre-coated silica gel G60 using CAMAG Linomat 5 instrument and the bands were detected under UV 366nm. [22,23]

Cytotoxicity test for plant extracts

Prior to assessing the inhibitory effects of the plant extracts on viper venom, their in-vitro cell cytotoxicity levels were checked using the MTT assay. [24-26] The MTT assay was carried out on Baby Hamster Kidney (BHK) cell lines to determine the CC50 concentration of the extract. It is the concentration of the plant extract at which 50% of the BHK cells are killed. All the later tests that were performed were done with a concentration of the plant extracts below the cytotoxicity level. [27,28]

Inhibition of Caseinolytic activity

Different concentrations of agar and casein were tried out on a trial error basis, and it was decided that the agar concentration to be used was to be 2.5% and that of casein was 1%. [29,30] For standardization experiments, 9mm wells were punched in the 'casein agar' plates and different concentrations Russell's viper venom (50 µl) were loaded into them. The plates were then kept for an overnight incubation at 37°C. Zone diameters were checked and the one which showed a constant gradation with time was selected for the test experiments. In test experiments, constant amounts of venom (which was selected from the standardized experiment) were mixed with different concentrations of plant extracts in 1: 1 ratio and the mixtures were incubated for an hour before loading them into the wells in the agar plates. They were then kept for overnight incubation at 37°C. Positive controls for both venom and plant extracts were used. After incubation, all the plates were seeded with 5% Trichloroacetic acid and the resulting zones were visualized after 10 minutes and measured. The effective plant dose which reduced the diameter of the precipitation zone by 50% as compared to diameter of zone induced by the dose of venom alone was selected. The recorded data was statistically analyzed using 'one tailed T-Test'.

Inhibition of Phospholipase A2 activity

Phospholipase A2 activity was measures using kit based PLA_2 Assay based on the method described by Burke *et al.* 2009 [31-33] Phospholipase A2 (PLA_2) catalyzes the hydrolysis of phospholipids at the *sn*-2 position yielding a free fatty acid and a lysophospholipid. The release of arachidonic acid from membrane phospholipids by PLA_2 was believed to be a key step in the control of eicosanoid production within the cell. In this assay we have substituted the $sPLA_2$ present in Russell's viper venom by the $sPLA_2$ present in bee venom. Though $sPLA_2$'s will have different structures in different species all of them catalyze the same reaction, i.e. the hydrolysis of phospholipids at the *sn*-2 position. Since all of them show the same mechanism of action we substitute bee venom PLA_2 for snake venom PLA_2. We are of the opinion that if the plant extracts do have an inhibitory effect on the bee venom PLA_2 they might also inhibit the PLA_2 present in snake venom. The recorded data was statistically analyzed using 'one tailed T-Test. [34-36]

Inhibition of procoagulant activity

The assay for procoagulant activity was performed according to the method described by Theakston and Reid; various concentrations of venom (100µl) were added to 200µl of human citrated plasma at 37°C. Coagulation time was recorded and the minimum coagulant dose (MCD) was determined as the venom concentration which induced clotting of plasma within 60 seconds. Plasma incubated with PBS alone served as the control. In neutralization assays, constant amount of venom was mixed with various dilutions of plant extracts in 1:1 ratio. The mixtures were incubated for 30mins at 37°C. Then 100µl of the mixture was added to 200µl of human citrated plasma and the clotting timed

recorded. In control tubes, plasma was incubated with either venom or plant extracts alone. Neutralization was expressed as effective dose (ED), defined as the concentration of plant extract at which clotting time increased three times when compared with clotting time of plasma incubated with MCD dose alone. [37,38]

RESULTS

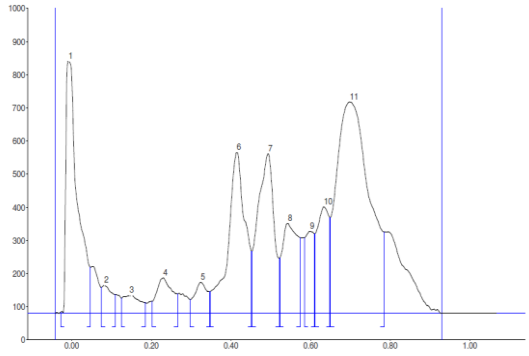
Preliminary phytochemical screening

Preliminary phytochemical studies revealed the presence of alkaloids, Glycosides, saponins, phytosterol and flavonoids in cold extract of leaves of *Hibiscus rosa-sinensis whereas.* Alkaloids, Glycosides, saponins and Phenols were detected in the cold extract of rhizomes of *Acorus calamus*. The cold Methanolic extracts were subjected to phytochemical screening for its constituents by standard methods and the results are tabulated in Table no 1.

Table No 1: Preliminary Phytochemical Screening

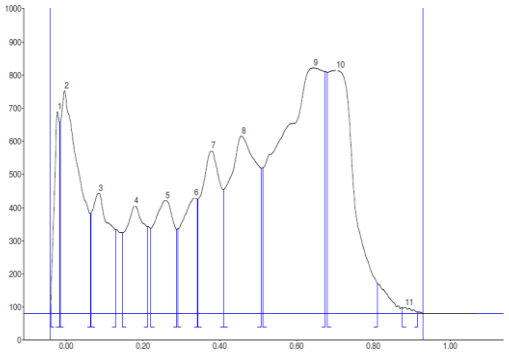
Plant constituent	*Hibiscus rosa-sinensis*	*Acorus calamus*
Alkaloids	+ +	+ +
Glycosides	+	+
Saponins	+ +	+ +
Phytosterols	+	-
Tannins	-	-
Phenols	-	+
Flavonoids	+	-

Assessment of the phytochemical constituents of the extracts using HPTLC



Graph 1: HPTLC of Ethanolic extract of *Hibiscus rosa-sinensis* (*Eleven* compounds were separated having R_f values -0.01, 0.08, 0.15, 0.23, 0.32, 0.42, 0.49, 0.54, 0.60, 0.63, 0.70)

Preliminary phytochemical constitutes were also studies using HPTLC



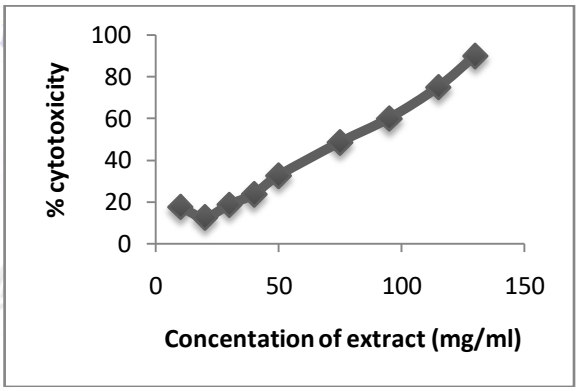
Graph 2: HPTLC of Methanolic extract of Acorus calamus (Eleven compounds were separated having R_f values -0.02, 0.00, 0.08, 0.18, 0.26, 0.33, 0.38, 0.46, 0.65, 0.71, 0.88)

Cytotoxicity test for plant extracts

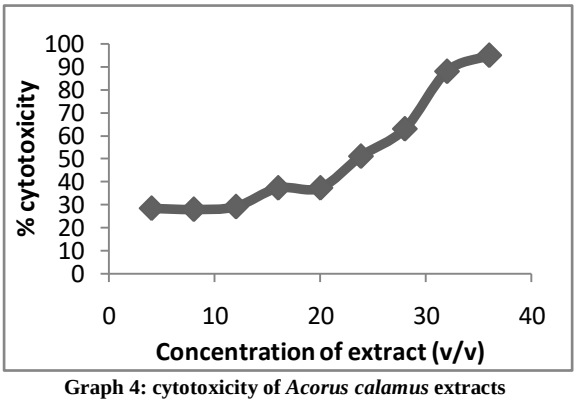
The CC50 values of the plant extracts are tabulated in table no 2.

Table No. 2: cytotoxicity test of plant extracts

Plant extract	CC50
Hibiscus rosa-sinensis (ethanolic extract)	75mg/ml
Acorus calamus (Methanolic extract)	24%(v/v)



Graph 3: Cytotoxicity of *Hibiscus rosa-sinensis* extracts



Graph 4: cytotoxicity of *Acorus calamus* extracts

Inhibition of Caseinolytic activity

The Russell's viper venom (50% v/v) which produced clear zone diameters of 17.5 + 0.5 mm on the Casein agar plates were selected as the initial caseinolytic doses. The following figures demonstrate the effect of the ethanolic and methanolic

extract of *Hibiscus rosa-sinensis* and *Acorus calamus* on the caseinolytic potential of viper venom respectively. The effectiveness was seen in a dose dependent manner. The maximum inhibition of the caseinolytic activity was seen at a concentration of 70mg/ml (w/v) of *Hibiscus rosa-sinensis* extract.

Table 3: Zone diameters for *Hibiscus rosa-sinensis* extracts

Concentration →	70mg/ml	60mg/ml	50mg/ml
Zone diameter ↓	16mm	15mm	17mm
	15.5mm	17mm	17mm
	14mm	17mm	17.5mm

Table 4: Zone diameters for *Acorus calamus* extracts

Concentration →	20%	15%	10%
Zone diameter ↓	17mm	17mm	17mm
	15.5mm	16.5mm	17mm
	14 mm	15mm	16mm

Table 5: Standard deviation values for the respective concentrations of plant extracts

H70mg/ml	H60mg/ml	H50mg/ml	A20%	A15%	A10%
1.0408	1.1547	0.2887	1.8857	1.0408	0.5774

Table 6: Standard error values for the respective concentrations of plant extracts

H70mg/ml	H60mg/ml	H50mg/ml	A20%	A15%	A10%
0.7359	0.8164	0.2041	1.0606	0.7359	0.4082

Table 7: $|T|$ values for the respective concentrations of plant extracts

H70mg/ml	H60mg/ml	H50 mg/ml	A20%	A15%	A10%
3.1797	1.4331	1.6658	1.8857	1.8208	2.0406

Key: H- *Hibiscus rosa-sinensis*; A- *Acorus calamus*

The tabulated value for T5% d.f 2 is 2.902. From the calculated values it is seen that only *Hibiscus rosa-sinensis* at a concentration of 70mg/ml shows a $|T|$value greater than the tabulated one. Hence, a 'significant' negating potential against the caseinolytic activity of Russell's viper venom is shown only by Hibiscus and at the highest permissible concentration of 70mg/ml.

Inhibition of phospholipase A2 activity

$sPLA_2$ activity is the least in the sample with the highest concentration of plant extract as compared to just the control (which contained only bee venom PLA_2). It is also evident that as the concentration of the plant extract reduces, the PLA_2 activity is inching closer to that of the control.

Table 8: sPLA2 activity of Hibiscus rosa-sinensis

$sPLA_2$ of P.C	$sPLA_2$ of H70	$sPLA_2$ of H60	$sPLA_2$ of H50
0.1107	0.04365	0.043	0.0934

Table 9: sPLA2 activity of Acorus calamus

$sPLA_2$ of P.C	$sPLA_2$ of A20	$sPLA_2$ of A18	$sPLA_2$ of A16
0.1107	0.03825	0.0486	0.1095

Key: $sPLA_2$: $sPLA_2$ activity (µmol/min/ml)

Inhibition of procoagulant activity

The minimum coagulant dose (MCD) was determined as the venom dose inducing clotting of plasma in 60 seconds. About 1.635µg (12µl) of Russell's viper venom clotted human citrated plasma within 60 seconds. In the neutralization assay, the absence of clot formation shows the neutralizing activity of the plant extract. It was found that 0.84mg of Hibiscus rosa-sinensis extract was able to completely neutralize the coagulant activity. Acorus calamus extracts on the other hand didn't show any neutralizing capability even at the highest permissible dose. The absence of clot formation is visible in the following pictures:

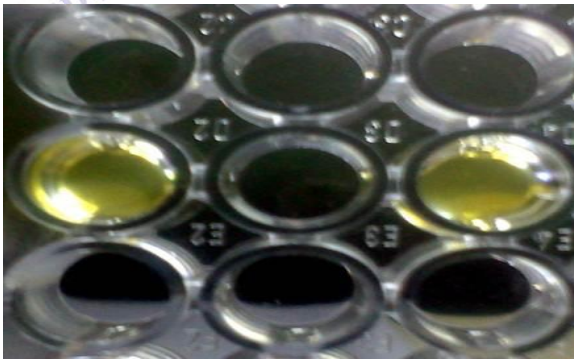


Figure 4: *Acorus extract* (20% and 18%)

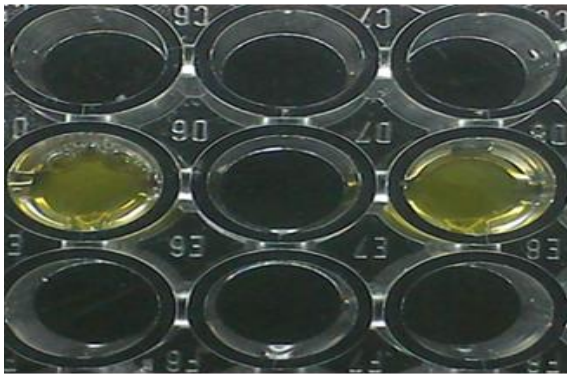


Figure 5: Hibiscus Extract (70mg/ml and 60 mg/ml)

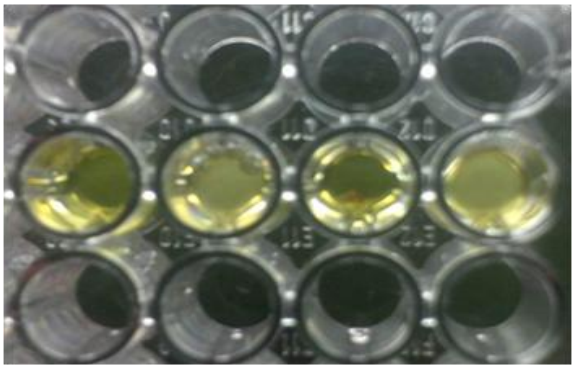


Figure 6: Venom and Plant controls

DISCUSSION

Snakebites being a major public health problem claim a large number of lives in the Indian sub-continent. Even after years of research, the most efficient treatment for snake envenomation is the specific heterologous serum. Also, the development of this Antivenom serum from an animal source is expensive and time consuming. Although, the use of plants against the effects of snake bite has been long recognized, more scientific attention has been given only in the last 20 years or so. Many Indian plants are recommended for the treatment of snake envenomation. [7, 9 ,11]

In the present study we checked the antivenom potential of Acorus calamus (rhizome) and Hibiscus rosa-sinensis (leaves) extract against Russell's viper venom. It is essential to understand the pharmacological action of snake venom in order to devise a rational treatment for snakebites. Pharmacological activities like caseinolytic activity, procoagulant activity and phospholipase (PLA2) activity of the viper venom were carried out in in-vitro conditions. Neutralization of these pharmacological activities was carried out using the above mentioned plant extracts. These studies were performed by incubating the venom with plant extracts prior to testing the effects (pre-incubation method).

The plant extracts were phytochemically screened by a variety of tests including HPTLC to determine the type and number of primary and secondary plant constituents. Many of these plant metabolites have been documented by researchers as having an inhibitory effect on snake venoms. Prior to assessing the inhibitory effects of the plant extracts on viper venom, their in-vitro cell cytotoxicity levels were checked using the MTT assay. The inhibition of the caseinolytic activity was evident from the pictures. Procoagulant activity induced by the viper venom was studied using human citrated plasma. [31] While Acorus calamus extracts failed to show any visible neutralizing effects *Hibiscus rosa-sinensis* was effective in antagonising the procoagulation effects of the venom. Researchers have suggested that the plant extracts causing inhibition of this enzymatic activity have compounds that bind to divalent metal ions (like Ca2+), which are required for enzymatic activities. At present we don't know the mechanism by which the plant extracts inhibited the PLA2 isolated from bee venom. But, if the suggested mechanism holds true, then our hypothesis would stand corrected and the plant extracts would definitely be able to inhibit the PLA2 present in viper venoms too. [35,36]

The so-called 'secondary metabolites' which are present in plants can be held responsible for the neutralizing effect of plants against the action of snake venoms, in popular use. The many different chemical structures shown to occur in such plants are all capable of interacting with macro molecular targets. Snake venoms are of a highly complex nature, made up of peptides and proteins. Many of these components are enzymes. The mechanism of their actions, still incompletely understood, can in great part be attributed to the blocking of receptors- structures prone to chemical attacks. Other vulnerable sites are metal atoms, present in metallo-proteinases, where sequestering by chelation offers a plausible explanation for the inhibition of enzymes. This experimental work proved the effectiveness of the plant extract on viper venom but the mode of action could not be explained. For this,

further research work has to be carried out. [1,6]

CONCLUSION

Results of the present study conducted on the few important in vitro activities of venom reveal the presence of antivenom principles in the methanolic extracts of *Pongamia pinnata*, *Piper longum*, *Sapindus laurifolius* and *Adhatoda zeylanica*. The efficacy of these extracts also justifies the traditional use of herbal extracts in the treatment of snake bite. However, further research including the isolation of Antivenom compounds and in vivo assays is needed to establish these plants as a remedy for snakebite.

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