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Research Article

EVALUATION OF ANTIVENOM POTENTIAL OF ACHYRANTHES ASPERA LEAVES AGAINST SNAKE VENOM

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Abstract

The antivenom potential of aqueous (AE) and ethanolic extract (EE) of the leaves of Achyranthes aspera plant was evaluated using in vitro assays. Soxhlet extraction of the dried, powdered leaves of Achyranthes aspera was carried out. The neutralization of lethal toxicity induced by D. russelli venom was assessed using mice. Leaf extract effectively neutralized the russeli's viper venom induced lethality (LD50) of 11 Âµg with effective dose (ED50) of 0.3mg for AE and 1.5mg for EE. Phospholipase A2 present in snake venom was neutralized at a dose of 0.05mg and 0.06mg of AE and EE leaf extract respectively. Further procoagulant activity was carried out using Echis carinatus venom. The extracts also effectively neutralized the venom induced hemolysis in the concentration range of 50-100Âµg. HPTLC analysis done for the extracts showed Rf values indicating same constituents in both extracts. The results obtained demonstrate that leaf extract of Achyranthes aspera possess snake venom neutralizing capacity and can be used as an adjuvant for antivenom therapy.

Key Words: Achyranthes aspera ; russelli's viper ;Phospholipase A2, Echis carinatus venom HPTLC

1. Introduction:

Snakebite is major concern in rural areas of tropical countries. According to the survey carried out on the global burden of snake bite, the reports showed world incidence of snakebite ranging from 1.2 to 5.5 million snake bites per year [1]. In India alone 35000 to 50000 peoples die per year out of the two lakh cases reported [2-3]. Venomous snakebite is noted as a common acute medical emergency by the World Health Organisation. Among the 216 species of snakes available in India, four major venomous species responsible for fatality in India are Nag (*Naja naja*), *Bungarus caeruleus*, *Daboia russelli* and *Echis carinatus* [4].

Snake venom is composed of a complex mixture of enzymes, proteins, carbohydrates and other molecules. Enzymes include phospholipase (sPLA2), hyaluronidase, hemorrhagic metalloproteinases, procoagulent,neurotoxins and other proteolytic enzymes.[5]. Phospholipase A2 (PLA2) is active and induces several pharmacological disorders including anti-coagulant, hemolytic, neurotoxicity, myotoxicity, cardiotoxicity,and platelet effect. [6].

Anti snake venom immunotherapy (ASV) is the only available treatment for snake bite. However treatment with ASV may lead to adverse reactions ranging from early allergic reactions (pruritus, urticaria) to fatal anaphylaxis. The cost and the non availability of these formulations in the rural areas is the major issue. The alternative treatment available is the traditional folk medicine. Plants are reported to neutralize the action of venom in folk medicine [7]. However, only a few species have been scientifically investigated with very few reports on their active components isolated and characterized each structurally and functionally.

Achyranthes aspera is found as a weed reported as a number of reports regarding the use of various parts of *Achyranthes aspera* in different regions of India are available, like inflorescence and seed paste is applied on the wounds caused by snake

bite in the rural areas of Kanyakumari, Tamil Nadu, leaf juice is applied by the tribes of Madhya Pradesh locally to neutralize snake poison. Root powder is used as an antidote by the people in Uttar Pradesh. Whole plant extract is given orally once as antidote by the tribes of Rewa district, in Madhya Pradesh [8]. In the present study the anti-venom activity of the leaf extract of *Achyranthes aspera* against viper venom was explored.

2. MATERIALS & METHODS

2.1. *Plant collection*

Fresh leaves of *Achyranthes aspera Linn* were collected from Pimpri, Pune in the month of August 2015.The authentification of collected parts of *Achyranthes aspera* was carried out by Botanical Survey of India, Pune. (Specimen no-PRG-1)

2.2. *Venom & Chemicals*

Freeze-dried snake venom powder of R*uselli's viper* and *Saw scaled vipe*) was obtained from Haffkine Biopharmaceutical Corporation Limited, Pune and stored at 4ºC. The venom was dissolved in 0.9% saline solution & centrifuged at 2000 rpm for 10 min & the supernatant was used for the study. All other reagents & solvents were of analytical grade & were purchased from SD fine chemicals Research Laboratory Pvt Ltd/HiMedia, Mumbai.

2.3. *Animals*

Swiss albino mice (18-20 g) were maintained in the Animal Experimental Laboratory of Haffkine Biopharmaceutical Corporation Limited, Pune at room temperature of (25±2) ºC, relative humidity of (75±5) % & 12 h dark –light cycle. They were fed with standard animal pellets & allowed free access to water. Laboratory animal handling and experimental procedures were performed in according to the guidelines of CPCSEA and experimental protocol was approved by Institutional Animal Ethics Committee.

2.4. *Plant extraction*

The shade dried leaves were powdered and 100gm powder was placed in the soxhlet thimble to carry out extraction with distilled water(AE) and ethanol (EE) each by continuous hot percolation using soxhlet apparatus for 72 hrs. The extract was then concentrated using rotary vacuum evaporator below reduced pressure at 40ºC. Dried leaf extract was stored at 5ºC in air tight containers.

2.5. *Lethal toxicity (in vivo* studies)

The median lethal dose (LD_{50}) of *D. russelli* venom was determined by injecting varying dose of venom in 0.3ml of physiological saline solution into the tail vein of mice, using groups of 3-5 mice for each venom dose. The LD_{50} was calculated by recording the number of deaths occurring within 24hrs of venom injection. The anti-lethal potentials of *Achyranthes aspera* plant extracts were determined against $2LD_{50}$ of *D. russelli* venom. Various amounts of plant extracts (µl) were mixed with $2LD_{50}$ of venom and incubated at 37^0 C for 30 minutes and then doses of 0.3ml of each mixture was injected intravenously into the mice. 3-5 mice were used at each dose. Control mice received only the snake venom without plant extracts. The effective dose (ED_{50}) was then calculated from the number of deaths occurring within 24hrs of injection of the venom/antivenom mixture [9].

2.6. *In vitro assays for neutralization of snake venom*

The *in vitro assays* were carried out in triplicates and mean values were calculated.

2.6.1 Inhibition of phospholipase activity (Indirect hemolysis assay)

Phospholipase activity was measured by using indirect hemolytic assay on agarose-erythrocyte-egg yolk gel plate as explained by Gutierrez *et al*. [10].Various doses of Russell's viper venom (µg/ µl) were added into 3mm wells in agarose gels. (PH 8, 0.8% in PBS) containing 1.2% sheep erythrocytes, 1.2% egg yolk as a source of lecithin and 10mM $CaCl_2$. Plates were incubated at 37˚C for whole night and the diameters of the hemolytic halos were measured. The minimum indirect hemolytic dose (MIHD) corresponds to a venom dose, which produced a hemolytic halo of 11mm diameter. A fixed amount of venom and AE/EE extract of *Achyranthes aspera* were incubate and mixed together at 37^0C for 30 min. Aliquots of 10µl of the mixtures were added to the wells in agarose egg yolk-sheep erythrocyte gels. Control sample contains only venom. Plates were incubated at 37^0C for 20hrs.Neutralization was expressed as ratio of mg of extract to mg of venom required to produce 50

% of decrease in diameter of hemolytic halo when compared with the control sample.

2.6.2 .*Inhibition of Procoagulant activity*

The procoagulant activity was performed according to the method expressed by Reid and Theakston et al., and modified by Laing *et al.* [11,12].Various amounts of *Echis carinatus* venom dissolved in 100µl Phosphate buffer saline (PBS) solution (pH 7.2) was added to human citrated plasma at 37°C and coagulation time was recorded to determine the Minimum Coagulant Dose (MCD) of snake venom, which induced clotting of plasma within 60sec. A mixture of plasma with PBS served as control which was incubated at 37^{0}C. Mixture of minimum coagulant dose of venom with different dilutions of AE and EE and incubated at 37^{0}C for 30 min. Further, 0.1ml of the mixture was added to 0.3ml of citrated plasma and the clotting time of plasma was recorded. Control tubes contain either of venom or extract mixed with the plasma. Neutralization was expressed as ED (effective dose) given by ratio of µL of extract to mg of venom required to increase clotting time of plasma by 3 times when compared to clotting time of plasma incubated with two MCD venom alone.

2.6.3. *Inhibition of Hemolytic activity*

The hemolytic activity was performed according to the method described by Thushara et al., [13]. The hemolytic action of venom and plant *Achyranthes aspera* extract was studied *in vitro* by using RBCs. Citrated blood (5ml) was centrifuged for 10 min at 900 rpm. The supernatant was removed and the pellet was washed twice with physiological salt solution. For the normal control, 5 ml of physiological saline solution and 0.5 ml of RBCs mixture was taken in the tubes and for 100% hemolysis, 5ml of distilled H_20 mixed with 0.5ml of washed RBCs (positive control) was placed into the tubes. The test samples were prepared with 5ml of venom/extract with 0.5ml of washed RBCs. All the tubes were put in a thermostat for 1hour at 37°C and centrifuged it at 2000 rpm for 20min. The supernatant fluid was transferred to separate tubes to measure the optical density (OD) using spectrophotometer at a wave length of 540nm against water. The calculation for % hemolysis was done by the formula.

$$\frac{\text{OD of test sample} - \text{OD of normal control}}{\text{OD of 100\% hemolysis (positive control)}} \times 100$$

2.7. *Phytochemical analysis*

In order to obtain insight into the different phytochemicals present in the extract, analysis was performed as per the methods described by Kokate et al. [14] and Khandelwal [15].

2.8 High performance thin layer chromatography

2.8.1. Preparation of extract stock solution:

Aqueous extract solution: Accurately weighed quantity (5 mg) of AE transferred to 10.0 ml volumetric flask, added 2 ml of water and ethanol and ultra-sonicated for 10 minutes, volume was then made up to the mark with ethanol (50 µg/µl) and filtered it.

Ethanolic extract solution: Accurately weighed quantity (5 mg) of EE transferred to 10.0 ml volumetric flask, added 2 ml of ethanol and ultrasonicated for 10 minutes, volume was then made up to the mark with water (50 µg/µl).

2.8.2 **Selection of mobile phase**:

Aliquot portions of solutions A and B, 20 µl each, were applied on TLC plates in the form of band (band size: 6 mm) and the plates were run in different solvent systems. In an attempt to achieve resolved peaks with a compact band, several trials were made by using different solvent systems containing non-polar solvents and relatively polar like Toluene**:** ethyl acetate: methanol, n-Hexane: Ethyl acetate: Glacial acetic acid, Chloroform: toluene: Methanol, Toluene: ethyl acetate: formic acid in different proportion to obtain isolated peaks for various constituents present in the leaf extract. Among the different mobile phase combinations tested Toluene: ethyl acetate: formic acid (4.5:0.5:0.1v/v/v) gave desired peaks when scanned over the wavelength of 200-700nm.Maximum absorbance was observed at 254nm.

3. RESULT & DISCUSSION

In the present study, Soxhlet extraction of the dried, powdered leaves of *Achyranthes aspera* was carried out using water and ethanol as solvent. Various phytochemical constituents isolated from the plant are reported for their diuretic, hepatoprotective, antiallergic along with anti-inflammatory and antioxidant activity. Further survey on tradi-

tional medicine also indicated the use of plant against snake bite. [16, 17]. Therefore, in the present study, AE and EE were evaluated for their potential as enzyme inhibitors against the Viperidae family.

3.1. *Lethal Toxicity (in vivo* studies)

The lethal toxicity (LD50) of D. russelli venom was assessed using swiss albino mice. About 11 µg of D. russelli venom exhibited the lethal activity(LD50) for mice weighing 18-20g. The neutralization of lethality was assessed by mixing constant amount i.e.15 µg of venom with various dilutions of AE and EE incubated at 37°C for 30 minutes prior to injection. It was recorded that 0.3 mg of aqueous and 1.5 mg of ethanolic leaves extracts were capable to completely neutralize the lethal activity of *Ruselli Viper* venom.(Table 1).

Table 1 Neutralization of Ruselli viper venom induced lethality by Achyranthes aspera AE and EE

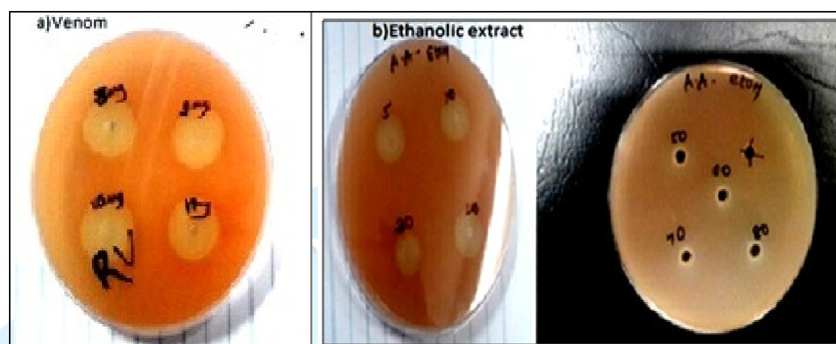
Plant Extract	Concentration of Ruselli viper venom (µg/µl)	Neutralization of Venom by plant extract (ED_{50} in mg)
AE	15 µg ($2LD_{50}$)	0.3 mg
EE	15 µg ($2LD_{50}$)	1.5 mg

Table 2 Inhibition of PLA_2 activity of *D. russelli* venom by AE and EE

Sr.no	Conc(µg/µl)	Halo diameter (AE)	Halo diameter (EE)
S1	5 µg	14mm	15mm
S2	10 µg	15mm	15mm
S3	20 µg	14mm	14mm
S4	30 µg	14mm	14mm
S5	50 µg	07mm	08mm
S6	60 µg	6.5mm	08mm
S7	70 µg	6.5mm	7.5mm
S8	80 µg	6.5mm	7.5mm
Venom control	-	16mm	16mm

(a) Hemolytic halo produced by Russell viper venom (b) AE of *A. aspera*

Fig 1. Inhibition of phospholipase activity by aqueous leaf extract of *A. Aspera*



(a) Hemolytic halo produced by Russell viper venom (b) EE of *A. aspera*

Fig 2. Inhibition of phospholipase activity by ethanolic leaf extract of *A. aspera*

Table 3 Determination of minimum coagulant dose (MCD) for *E. carinatus*.

Sr.no	Plasma	Venom (µg/µl)	Clotting time
1	150 µl	2 µg	42 sec
2	150 µl	2 µg	41 sec
3	150 µl	2 µg	38 sec
Mean clotting time			40 sec

Table 4 Determination of two minimum coagulant dose (2MCD) for *E. carinatus*.

Sr.no	Plasma	Venom (µg/µl)	Clotting time
1	150 µl	4 µg	30 sec
2	150 µl	4 µg	26 sec
3	150 µl	4 µg	28 sec
Mean clotting time		28 sec	

Table 5 Inhibition of procoagulant activity by extracts of *Achyranthes aspera* against E. *carinatus* venom

Sr. no	Extract(µg/µl)	Clotting time with AE	Clotting time with EE
1	1 µg	2 min 17sec	1min 38 sec
2	2 µg	2 min 35 sec	2 min 10 sec
3	3 µg	3 min 55 sec	2 min 59 sec
4	4 µg	4 min 12 sec	3 min 20 sec

3.2 *In vitro assays for neutralization of snake venom*

3.2.1 Phospholipase *Activity*

Phospholipases enzymes are multifunctional that degrade membrane phospholipids and release arachidonic acid which is a precursor in causing inflammation by cyclooxygenase (COX) or lipooxygenase (LOX) pathways. In phospholipase activity (PLA2), *Ruselli Viper* venom was able to produce hemolytic haloes in agarose-sheep erythrocytes gel. It is reported that 10µg of *Ruselli viper* venom produced 11mm diameter of hemolytic halo, which is considered to be 1U (U/10µg). However, in the present experimental work, venom showed 16mm diameter of haemolytic halo which indicated that *Ruselli viper* venoms have the enzymes (PLA2) that has the ability to lyse sheep RBC's. *Achyranthes aspera* Aqueous & ethanolic leaf extracts were capable of inhibiting PLA2 dependent hemolysis of sheep RBC's induced by the *Ruselli viper* venom in

a dose dependent manner. (Table 2). It was found that 50µg of AE and 60µg of EE could produce 50 % of decrease in diameter of hemolytic halo when compared with the control sample (10µg) venom (Fig 1 and 2). Terpenoids like oleanolic acid present in the plant extract is reported to inhibit phospholipase A_2 activities of the *ruselli's viper* venom.

3.2.2 *Procoagulant Activity*

The Minimum Coagulant Dose (MCD and 2MCD) of *Echis carinatus* was determined by testing various concentration of venom and it was found that 2µg of *Echis carinatus* venom could induce clotting of human citrated plasma within 60sec (Table 3) while the 2MCD amount of venom generated coagulation within 30sec (Table 4). In the neutralization assay, the absence of clot formation proved the neutralizing ability of both aqueous & ethanolic leaf extracts (Table 5) where in 1 µg of both the extracts delayed the clotting. The procoagulant activity demonstrated the ability of the plant extract to inhibit clotting caused by venom.

Table 6 Inhibition of haemolytic activity by AE and EE leaf extract of A*chyranthes aspera*

Conc of extract(µg/µl)	% hemolysis by AE	%Hemolysis by EE
50µg	32.83	22.73
60µg	26.62	10.86
70µg	20.91	6.57
80µg	7.39	6.11
90 µg	2.87	5.84
100 µg	2.60	1.41
+Ve control	96.80	
-Ve control	0	
Venom control	36.48	

Table 7 Qualitative analysis of the phytochemicals present in aqueous and ethanolic extract of *Achyranthes aspera*.

Phytochemicals	Aqueous extract	Ethanolic extract
Alkaloids	+	-
Flavonoids	+	+
Proteins	-	-
Carbohydrate	+	+
Tannins	+	+
Sterols	+	-
Glycosides	+	+
Phenols	+	+
Saponins	+	+
Terpenoids	+	+

3.2.3. *Hemolytic activity*

Hemolytic activity is another distinct feature of *Ruselli viper* venoms greatly induced by metalloproteases, PLA2, and specifically, cardiotoxins and cytotoxins of venom [18, 19, 20]. *Russelli viper venom* (*50*µg), showed lysis of RBCs and was considered as venom control and selected as the Minimum Hemolytic Dose (MHD). Different concentrations of aqueous and ethanolic extract of *Achyranthes aspera* leaf ranging from 50-100 µg were used for the study to neutralize the hemolysis of RBCs produced by *D.russelli* venom. It was observed that water which served as positive control gave 96.80% hemolysis while the Ruselli venom

produced 36.48% hemolysis. The percentage hemolysis calculated for the AE and EE leaf extract was 2.60 % and 1.41 % respectively at a dose of 100µg (Table 6). It is possible that the phytochemicals present in the leaf extract inhibit toxic proteins of the venom in a dose dependent manner.

3.3. *Phytochemical analysis*

Aqueous & ethanolic extraction of the *Achyranthes aspera* leaves was carried out. The phytochemical tests revealed the presence of various active constituents as mention in (Table 7). Flavonoids, polyphenol & terpenoids are the secondary metabolites present in leaf extract, reported for protein binding and enzyme inhibiting properties [21]. Oleanolic acid present in *Achyranthes aspera* is reported as potent inhibitor against snake venom containing metalloprotease and phospolipase A_2 as the key enzymes [22].

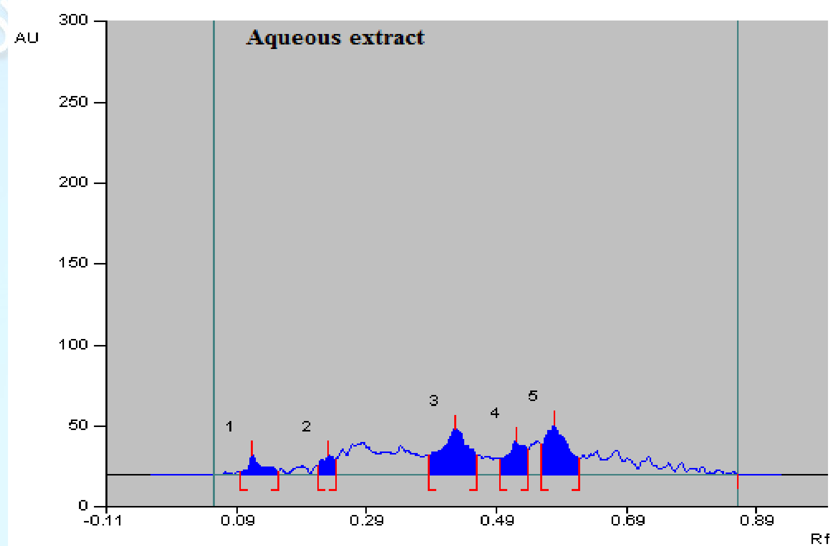


Fig 3. Densitogram of Aqueous extract of *Achyranthes aspera*

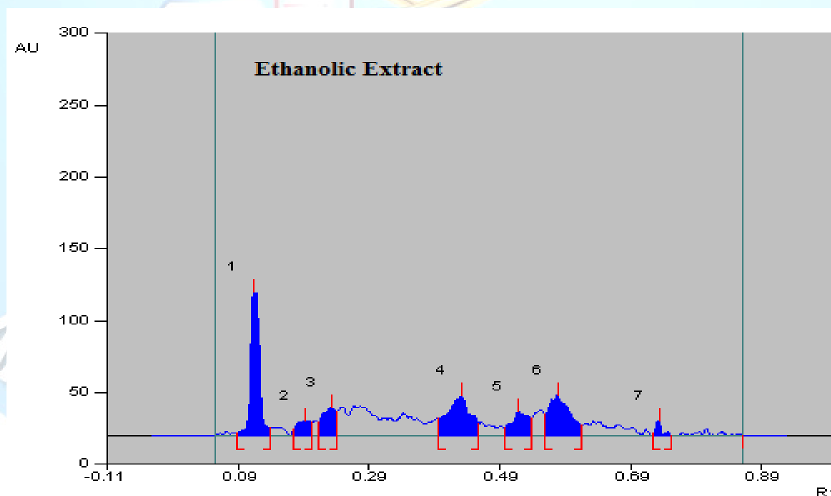


Fig 4. Densitogram of ethanolic extract of *Achyranthes aspera*

Table 8. R_f value of AE

Peak	R_f value forAE	Area
1	0.11	224.6
2	0.23	203.5
3	0.42	999.8
4	0.52	486.6
5	0.58	891.1

Table 09. R_f value of EE

Peak	R_f value for EE	Area
1	0.11	1161.5
2	0.19	180.9
3	0.23	340.5
4	0.43	790.7
5	0.52	365.9
6	0.58	748.5
7	0.73	71.7

3.4. *High performance Thin layer Chromatography*

The HPTLC analysis done for aqueous and ethanolic leaf extracts of *Achyranthes aspera* using Toluene: ethyl acetate: formic acid (9:1:0.1v/v/v) showed five peaks and seven peaks respectively which were well resolved. According to HPTLC method developed by S.Sindhu et.al [23] for the estimation of oleanolic acid using hexane-ethylacetate-acetic acid (8:4:0.2v/v/v) as mobile phase, oleanolic acid resolved at 0.53 Rf value (Figure 3 and 4). The leaf extracts show peak with the R_f value 0.52 at 254nm suggesting the presence of oleanolic acid. Peaks at Rf value 0.11, 0.23, 0.52 and 0.58 were common while aqueous extract also had additional peak at Rf 0.42 ad ethanolic extract showed peaks at 0.43 and 0.73. (Table 8 and 9).

5. *Conclusion*

Many developing countries depend on the use of herbal medicines against the severe patho-physiological conditions of a snake bite. *Achyranthes aspera (Amaranthaceae)* is an important medicinal herb found as a weed throughout India reported for its use against snake bite. The present experimental results indicate that aqueous & ethanolic extract of *Achyranthes aspera* leaves were effective in neutralizing the main toxic and enzymatic effects of snake venom of Viperidae family. The antivenom properties of aqueous extracts exhibited better potency when compared to ethanolic extract to neutralize the lethality and inactivate other pharmacological effects of venom. Further identification and purification of the active components of the plant invlved in the neutralization of the snake venom can be done.

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