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Effects of Aqueous Stem Extract of *Achyranthes aspera* on *Bitis arietans* Venom Protease and Phospholipase A_2 Activity

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Abstract: Aqueous stem extract of *Achyranthes aspera* was investigated for inhibitory activity against *Bitis arietans* venom protease and phospholipase A_2 activity. The elemental analysis and phytochemical screening of the plant extract were carried out. The activities of protease and phospholipase A_2 (V_o) of the crude *Bitis arietans* venom was determined and the data obtained was used to estimate K_M, V_{max} and K_{cat}. Inhibition studies were carried out using the same procedure except that different concentrations of the extracts (5%, 10%, 15% for protease assay and 0.5%, 0.75%, 10%, 1.25% and 1.5% for phospholipase A_2 assay) were added to the reaction mixture. The result showed that the *Bitis arietans* venom protease had a V_{max} of 0.062 ± 0.013 µmol/min, K_M of 0.496 ± 0.095mg/ml and a K_{cat} of 0.125 ± 0.001min^{-1}. The result also indicates that the *Bitis arietans* phospholipase A_2 had a V_{max} of 3.27 ± 0.030min^{-1}, K_M of 8.358 ± 0.050 mg/ml and K_{cat} of 0.391 ± 0.002min^{-}. The aqueous stem extract produced a statistically significant ($P<0.05$) decrease in the V_{max}, K_M and K_{cat} of the *Bitis arietans* venom phospholipase A_2 in a dose dependent manner and a statistically significant ($P<0.05$) increase in the V_{max}, K_M and K_{cat} of *Bitis arietans* protease in a dose dependent manner. The phytochemical screening revealed the presence of flavonoids, tannins, steroids, saponins and terpenoids in the extract while the elemental analysis revealed the presence of Zn, Cr, Ni, Cd, Mn, Fe and Na. The result suggests that aqueous stem extract of *Achyranthes aspera* inhibited the *Bitis arietans* venom phospholipase A_2 in an uncompetitive manner while the protease activity was stimulated by the extracts. It was observed that the use of the stem of *Achyranthes aspera* may be important in the treatment of snake bites.

Keywords: *Achyranthes aspera, Bitis arietans*, Antivenom, Protease, Phospholipase A_2

1. Introduction

Bitis arietans (puff adder) belongs to the family viperidae and is one of the most dangerous snakes found in Africa. [10]. Viper venom contains protease and phospholipase A_2. Snake venom phospholipase A_2 have injurious effects such as haemolysis of red blood cells, anticoagulation and cardiotoxity. Snake venom proteases on the other hand is responsible for the severe bleeding observed in snake bite victims, interference with blood coagulation and haemostatic plug formation and degradation of the extracellular matrix components of the victims of snakebite [21]. These two enzymes therefore having implicated in such a variety of pathological mechanisms can be said to play a central role in the pathology of *Bitis arietans* envenomation. Therefore possible blockage or inhibiting of their action could unveil a way of ameliorating or totally rendering ineffective the toxicity posed by the venom. *Achyranthes aspera* belonging to the family Amaranthacea is claimed to be used in the North Eastern parts of Nigeria in the treatment of snakebite. Hence this study investigated the invitro effect of the aqueous stem extract on *Bitis arietans* venom protease and phospholipase A_2 activity.

2. Materials and Method

2.1. Chemcals

All the chemicals used in this study were of analytical grade and purchased from various sources.

2.2. Snake Venom

Freeze dried *Bitis arietans* Snake Venom was obtained from the Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria, Nigeria.

2.3. Plant Material

Fresh stems of *Achyranthes aspera* were collected from Biu, Borno State Nigeria. The voucher number was obtained and stored in the herbarium. It was washed and shade dried for two weeks to a constant weight. The dried stems were pounded to fine powder with mortar and pestle.

2.3.1. Extract Preparation

One hundred grams of the plant material was transferred to two liter of round bottom flask containing One liter of water. The condenser was fitted to the flask. The flask and the material were heated for 45 minutes. The solution was decanted to remove debris. This was repeated three times. The filtrate was poured onto an evaporating dish concentrated on a water bath. The extract was transferred to airtight containers for further analysis.

2.3.2. Phytochemical Screening

The presence of anthraquinone, combined anthraquinone, steroidal nucleus, terpenoids, saponin, glycosides, flavonoids, alkaloids, tannins were tested as described by Sofowora, [23],Harborne [11], Trease and Evans [24].

2.3.3. Elemental Analysis

The mineral composition of the extract was determined using UV-spectrometer with computer readout after acid digestion [4].

2.4. Protease Assay

The protease activity was assayed as described by Fahmey *et al,* [8]. Briefly, 50µl of the crude venom solution (10 mg/ml) was incubated with 500µl of 100mM sodium acetate buffer, pH 4.5, and 100ul of 3% Casein at 37°C. The mixture was made up to 1ml with distilled water. Assays were carried out after 1hr, the reaction was stopped by the addition of 200µl of 20% trichloroacetic acid. This was followed by the removal of the precipitated proteins by centrifugation at 10,000g. The absorbance of the supernatant was measured at 366nm. The activity of the protease is defined as the amount of enzyme that hydrolyses 1µmol of amino acids (in terms of tyrosine) from casein per minute under the standard assay conditions.

2.5. Phospholipase A_2 Assay

This was carried out by modification of the method of Haberman and Neumann as described by Okonogi *et al,* [17] .Here 0.5ml of egg yolk suspension (2 mg mL^{-1}) was introduced into a clean test tube containing 50µl of 1mM $CaCl_2$. To this, 100µl of 20 mg ml^{-1} venom solution was added and incubated at 37°C for 1hr. Thereafter, the enzymes was stopped by heating at 100°C for 2 minutes, a drop of phenolphthalein was added and then titrated against 2mM NaOH solution to an end point. The same procedure was carried out in the absence of the enzyme in order to obtain titre value for the blank for adequate comparison to deduce effect of the enzyme on the yolk (deduction of any FFA released). The activity of phospholipase A_2 was defined a the amount of enzyme required to hydrolyze 1mg of FFA from the lecithin present in the egg yolk under the standard conditions.

2.6. Determination of K_M, V_{Max} and, K_{cat}

The activities of protease and phospholipase A_2 (V_0) was determined in the presence and absence of various concentrations 5%, 10%, 15% for protease and 0.5%, 0.75%,1.0%, 1.25% and 1.5% for phospholipase A_2 assay) of the plant extracts. Data obtained was used in estimating the K_M, V_{Max} and, K_{cat}

2.7. Statistical Analysis

The Data obtained was presented as mean± standard deviation and analysis of variance was used to compare paired means and a difference was considered statistically significant $p<0.05$.

3. Results

Table 1. *Phytochemical screening of aqueous stem extract of Achyranthes aspera.*

Phytochemical	Presence/Absence
Alkaloid	-
Flavonoid	+
Tannins	+
Steroid	+
Saponins	+
Phenolic group	-
Terpenoids	+
Anthraquinone	-

Key.
+ Present.
- Absent.

Table 2. *Elemental analysis (ppm) of aqueous stem extract of Achyranthes aspera.*

Element	Concentration
Zn	0.07± 0.02
Cr	0.05± 0.02
Ni	0.71 ± 0.30
Cd	0.07± 0.50
Pb	-
Mn	0.20± 0.10
Fe	0.45 ± 0.10
K	8.50 ± 0.20
Na	114.68±10.10

Key- Absent.
Values are mean ±standard deviation for triplicate determinations.

Table 1 shows the results of phytochemical screening of the aqueous stem extract of *Achyranthes aspera*. The result shows the presence of flavonoids, tannins, steroids, phenolic group and terpenoids, while alkaloids and anthraquinones were absent.

Table 2 shows the results of elemental analysis of aqueous stem extract of *Achyranthes aspera*. The result shows that Zn, Cr, Ni, Cd, Pb, Mn, Fe, K and Na are present in the aqueous stem extract and *Achyranthes aspera* at varying concentrations with Na accumulating at highest level while Pb was not detected.

Table 3. *Effect of different concentrations of aqueous stem extract of Achyranthes aspera on Bitis arietans venom protease activity.*

Kinetic Parameters	Concentration of Extracts			
	Control	5%	10%	15%
K_M (mg/ml)	0.496±0.095	1.210 ± 0.110^c	4.690 ± 0.310^c	5.650 ± 0.750
V_{max} (µmol/min)	0.062 ±0.013	0.660 ± 0.160^c	04.740± 0.950^c	10.100 ± 0.300
K_{cat} (min^{-1})	0.125 ±0.001	0.5454 ± 0.011^c	1.010± 0.064^c	1.788± 0.020

Values are mean + SD of replicates.
Values with different superscript letters within row are significantly different from each other (P<0.05).

The result of the effect of aqueous stem extract of *Achyranthes aspera* on *Bitis arietans* protease activity as shown in Table 3 shows that the aqueous stem extract of *Achyranthes aspera* produced a dose dependent increase in the computed physiological index of efficiency of *Bitis a rientans* venom protease. The Michealis Mentens (Km) and maximum velocity (V_{max}) of *Bitis arientans* venom protease were all significantly increased in the presence of the extract.

Table 4. *Effect of different concentrations of aqueous stem extract of Achyranthes aspera on Bitis arietans phospholipase A_2 activity.*

Kinetic parameters	Concentration of Extracts					
	Control	0.5%	0.75%	1.0%	1.25%	1.5%
K_M(mg/ml)	8.358±0.050	6.500±1.170^b	4.55±1.040^c	4.040±0.520^c	3.500±0.500^c	2.002±0.140^c
V_{max}(µmol/min)	3.270±0.030	2.500±0.400	1.400±0.300^c	1.230±0.100^c	1.010±0.010^c	0.40±0.070^c
K_{cat} (min^{-1})	0.391±0.002	0.385±0.020	0.308±0.013^c	0.304±0.043^c	0.289±0.001^c	0.249±0.005^c

Values are mean ± SD of 3 replicates. N=3.
Values with different superscript letters within a row are significantly different from each other (P< 0.05).

The result of the effect of the aqueous stem extract of *Achyranthes aspera* on *Bitis arietans* venom phospholipase A_2 shows that the Michealis Mentens constant (Km) and the maximum velocity (V_{max}) of the *Bitis arietans* venom phospholipase A_2 were significantly decreased in the presence of the aqueous stem extract of *Ahyranthes aspera* and thus the computed physiological index of efficiency (K_{cat}) also decreased in the presence the extract. This suggests a Classical uncompetitive inhibition.

4. Discussion

The presence of flavonoids,tannins,steroids,saponins and terpenoids in the aqueous stem extract of *Achyranthes aspera* revealed in this study corroborates with the findings of Dey [6] who reported that *Achyranthes aspera* plant contains sterols, alkaloids, saponins, flavonoids and terpernoids .

Again,the presence of Zn, Cr, Ni, Cd, Mn, Fe, Kand Na in the aqueous stem extract of *Achyranthes aspera* as revealed in this study corroborates with the findings of Jabeen [13] who reported that *Achyranthes aspera* plant contains among others elements Zn, Cr, Ni, Cd,Pb Mn, Fe, K, Na, and Mg. Shendkar *et al* [22] reported that the aqueous stem extract of *Achyranthes aspera* contained Na, Cr, Ni, Zn, Pb, Mn, and Fe. Although Pb was found to be absent. Their analysis indicated a higher concentration of K. The presence of Na and K in the extracts implies that the plant may be of help in maintaining electrolyte balance in the body. Richards *et al* [19] reported that high salt concentration increases the activity of the HIV-1 protease. The addition of salt primarily affects the K_M value and the increase in proteolytic activity is usually attributed to the "salting out" of the hydrophobic substrate in the enzyme binding cleft. The increase in protease activity K_{cat}/K_M might be due largely to the concentrated presence of K^+ and also to the presence of Na^+. Sodium is more strongly attached to the protein surface primarily due to stronger interactions with carboxylate side chain groups of aspartates and glutamates. These effects are of particular importance for amino acid residues at or near the active site of the enzyme, including a pair of aspartates at the entrance of the reaction cavity. The entrance of binding site is occupied by negatively charged residues. Interactions of these charged residues with Na/K cations can modulate the electrostatic potential of the protease surface at the active site entrance and therefore influence substrate /inhibitor recognition [25]

The result of the effect of the aqueous stem extract of *Achyranthes aspera* on *Bitis arietans* venom protease activity as presented in Table 3 shows that the aqueous stem extract of *Achyranthes aspera* produced a dose dependent increase in the computed physiological index of efficiency of *Bitis arietans* venom protease activity. This indicates an increase in the number of casein molecule hydrolyzed to product at the saturation of the enzyme. This suggests an increase in the amount of protein degradation by *Bitis*

arietans venom protease activity. Some researcher previously reported that the aqueous root extract of *Achyranthes aspera* incorporated in the experimental diet of *Labeo rohita* significantly enhanced the serum antiproteases level than the fishes fed with control diet which did not contain the extract [18].

The result of the effect of the aqueous stem extract of *Achyranthes aspera* on *Bitis arietans* phospholipase A_2 activity shows an uncompetitive pattern of inhibition. The aqueous stem extract of *Achyranthes aspera* produced a dose dependent decrease in the computed physiological index of efficiency of *Bitis arietans* phospholipase A_2 activity which indicates a reduction in the number of free fatty acid hydrolyzed from the lecithin present in the egg yolk suspension. This is consistent with the report by Samy *et al,* [20] that the plant extract of *Achyranthes aspera* (glycosides) have shown potent snake venom neutralizing activity. The plant extract and partially purified fractions were administered orally to rats envenomed with rattle snake venom. Significant protection against venom induced changes in serum superoxide dismutase and Lipoprotein X levels were seen after administration of purified fractions.

Enzyme inhibiting and protein binding properties have been associated with chemically active compounds of flavonoids, polyphenols, terpenoids, xanthenes etc. These phytochemicals also inhibits phospholipase A_2 activities of both viper and cobra venom. Phenolics especially polyphenols like some tannins bind proteins acting upon component of venom directly and disabling them to act upon the receptors and according to Lans *et al* , [15]. They could also act by competitive blocking of the receptors.

Herbal constituents active against snake envenomation include among others alkaloids, steroids, tannins and terpenoids [9]. Several plant constituents including flavonoids and terpenoids possess protein binding and enzyme inhibiting properties and also inhibits snake venom phospholipase A_2 of both viper and cobra venom [3]. Polyphenols and tannins are attributable to reduction in enzyme activities [1]. Okonogi *et al*, [17] suggested that tannins in addition to other plant constituents which are known to unspecifically inactivate proteins to be the likely mechanism involve in detoxifying snake venom. Tannins precipitate proteins and form dark coloured complexes with metals such as iron [7]. Tannins are problems as they unspecifically bind to proteins and thereby may show non-specific (false positive) activity in enzyme assays [14].

Therefore the aqueous stem extract of *Achyranthes aspera* could serve as a good source of *Bitis arietans* antidote and could as well help in designing a novel drug to be used as an antivenin.

5. Conclusion

The stem extract of Achyranthes aspera possess potent snake venom neutralizing capacity and may provide protection against the toxicity posed by *Bitis arietans* venom hence it may be used for therapeutic purposes in case of snakebite.

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