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Effects of Aqueous Leaf Extract of *Achyranthes aspera* on *Bitis arietans* Venom Protease and Phospholipase A_2 Activities

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Abstract— **Aqueous leaf 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 was carried out. The activities of protease and phospholipase A_2 (V_o) of the crude *Bitis arietans* venom were 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 $\pm$ 0.013 µmol/min,K_M of 0.496 $\pm$ 0.095mg/ml and a K_{cat} of 0.125$\pm$ 0.001min^{-1}. The result also indicates that the *Bitis arietans* phospholipase A_2 had a V_{max} of 3.27 $\pm$ 0.030min^{-1}, K_M of 8.358 $\pm$ 0.050 mg/ml and K_{cat} of 0.391$\pm$ 0.002min^{-1}. The aqueous leaf extract produced 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 leaf 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 leaf of Achyranthes *aspera* may be important in the treatment of snake bites.**

Index Terms— ***Achyranthes aspera, Bitis arietans*, antivenom, protease. phospholipase A_2.**

I. Introduction

Highlight Snake bite remains a major public health and agricultural problem throughout the world, particularly Africa. It affects man and his domestic animals causing specific problems (cardiotoxic, neurotoxic) and untimely death (Aguiyi, 2011).

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Antivenom immunotherapy is the only specific treatment against snake venom envenomation. There are various side effects of antivenom such as anaphylactic shock, pyrogen reaction and serum sickness. In addition to this is expensive (Menatchisundaram*et al.,* 2009).

Bitis arietans(Puff adder) belonging to the family viperidae is one of the dangerous snakes in Nigeria, and is one of the commonest causes of envenomation in Northern Nigeria.

Snake venoms are composed of complex mixtures of active substances mainly peptides and proteins which are able to interfere with biological processes including thrombosis by affecting platelet aggregation and blood coagulation. Some of these proteins include enzymes like phospholipase A_2 and metalloproteases (Ibrahim *et al.,* 2011).

Achyranthes aspera which belongs to the family *Amarantheceae* is a perennial stiff, erect herb, growing up to 1-2m height and is claimed to be used in the treatment of snake bite in the north eastern parts of Nigeria especially Borno, Gombe and Adamawa. Hence this study intends to scientifically validate this claim by investigating the *invitro* inhibitory effect of the aqueous leaf extract of *Achyranthes aspera* on *Bitis arietans* venom protease and phospholipase A_2 activities.

II. MATERIALS AND METHOD

Location of the research

The study was conducted in the Department of Biochemistry, Faculty of Science, University of Maiduguri, PMB 1069, Maiduguri, Borno State, Nigeria.

Chemicals

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

Snake venom

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

Plant Material

The fresh leaves of *Achyranthes aspera* were collected from Biu, Borno State, Nigeria. This was authenticated by a plant taxonomist, Prof. S.S. Sanusi in Biological Sciences Department, University of Maiduguri, Borno State, Nigeria. The voucher number was obtained and deposited in the herbarium. The plant materials were washed and shade dried for two weeks to a constant weight. The leaves were pounded to fine powder using mortar and pestle and stored in clean dry

container until needed.

Extract preparation

One hundred grams of the plant material was transferred to 2 litres of round bottom flask containing 1litreof water. The condenser was fitted to the flask.The flask containing the material was 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

Phytochemical Screening

The presence of anthraquinone, combined anthraquinone, steroidal nucleus, terpenoids, saponin,glycosides, flavonoids, alkaloids, tannins were tested as descrbed by Sofowora, (1993); Harborne (1973); Trease and Evans(2002)

Elemental Analysis

The mineral composition of the extract was determined using UV-spectrometer with computer readout after acid digestion (AOAC, 1990).

Protease assay

The protease activity was assayed as described by Fahmey *et al,*(2004). Briefly, 50µl of the crude venom solution (10mg/ml) was incubated with 500µl of 100mM sodium acetate buffer, pH 4.5, and 100µl 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.

Phospholipase A_2 assay

This was carried out by modification of the method of Haberman and Neumann as described by Okonogi *et al,* 1979. 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 as the amount of enzyme required to hydrolyze 1mg of FFA from the lecithin present in the egg yolk under the standard conditions,

Determination of K_M,V_{Max},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},K_{cat}

Statistical analysis

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

III. Results

Table 1 Phytochemical screening of aqueous leaf extract of *Achyranthes aspera*

S/no.	Phytochemical	Presence/Absence
1	Alkaloid	_
2	Flavonoids	+
3	Tannins	+
4	Steroids	+
5	Saponins	+
6	Phenolic group	+
7	Terpenoids	+
8	Anthraquinone	-

KEY(S)

+	Present
_	Absent

Table 1 presents the result of Phytochemical screening of the aqueous leaf 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 Elemental composition (ppm) of Aqueous leaf extract of *Achyranthes aspera*

Element	Concentration
Zn	0.15 ± 0.02
Cr	0.09 ± 0.02
Ni	0.41 ± 0.20
Cd	0.17 ± 0.05
Pb	-
Mn	0.56 ± 0.20
Fe	0.45 ± 0.20
K	6.40 ± 0.30
Na	75.44 ± 0.10

Values are means ± standard deviation for Triplicate determination.

Key: Absent **-**

Table 2 shows the results of elemental analysis of aqueous leaf extract of *Achyranthes aspera.* The result shows that Zn, Cr, Ni, Cd, Pb, Mn, Fe, K and Na are present in the aqueous leaf 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 leaf 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	1.230 ± 0.011^b	1.360 ± 0.190^a	1.60± 0.110^c
V_{max} (µmol/min)	0.062 ±0.013	0.609 ± 0.110^c	0.709 ± 0.090^c	0.840 ± 0.150^c
K_{cat} (min^{-1})	0.125 ±0.001	0.4951 ± 0.007^c	0.521± 0.006^c	0.694 ± 0.010^c

Values are mean ± SD of triplicate determination

Values with different superscript letters within row are significantly different from each other (P<0.05).

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

Table 4 Effect of different concentrations of aqueous leaf extract of *Achyranthes aspera* on *Bitis arietans* venom 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 leaf extract of *Achyranthes aspera* on *Bitis arietans* phospholipase A_2 shows that the Michealis Mentens constant (K_m) and the maximum velocity (V_{max}) of the *Bitis arietans* venom phospholipase A_2 were significantly decreased in the presence of the aqueous leaf extract of *Achyranthes aspera* and thus the computed physiological index of efficiency (K_{cat}) also decrease in the presence of varying concentrations of the extracts.

IV. Discussion

The results of the effect of the aqueous leaf extract of *Achyranthes aspera* on *Bitis arietans* venom protease activity shows that the aqueous leaf extract of *Achyranthes aspera* produced a dose dependent increase in the computed physiological index of efficiency of *Bitis arietans* protease activity. This indicates an increase in the number of casein molecules hydrolysed to products at saturation of the enzyme. This may further suggest that the extract increased the amount of protein degradation by *Bitis arietans* venom protease activity. This implies that there was no inhibition of the protease activity.

The result of the effect of the aqueous leaf extract of *Achyranthes aspera* on *Bitis arietans* phospholipase A_2 activity shows an uncompetitive pattern of inhibition. The aqueous leaf extract of *Achyranthes aspera* produced a dose dependent decrease in the computed physiological index of efficiency. Therefore the aqueous leaf extract of *Achyranthesaspera* decreased K_M because of increased binding efficiency and decreased Vmax because they interfere with substrate binding and hamper catalysis in Enzyme substrate complex.Phospholipase A_2 activity which indicates a reduction in the number of free fatty acid hydrolysed from the lecithin present in the egg yolk suspension. Jabeen (2010) also reported that *Achyranthes aspera* plant contains among others elements Zn, Cr, Ni, Cd, Pb, Mn, Fe, K, Na, and Mg. 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.,* (1990) 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. Vrbka*et al.,* (2006) reported that 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. This is consistent with the report of Samy et al (2008) that the plant extract of *Achyranthes aspera* (glycosides) have shown potent snake venom neutralizing activity.

Gomes *et al.,* (2010) reported that herbal 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. Also Okonogi *et al.,* (1979) suggested that tannins in addition to other plant constituents which are known to unspecifically inactivate proteins to be likely mechanism involved in detoxifying snake venom.

Therefore the aqueous leaf 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.

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